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The present project had two major objectives. The first aim was to determine whether pyridostigmine would have differential effects on serum growth hormone in rats with differing cholinergic sensitivity. It was confirmed that the Flinders Sensitive Line (FSL) rats exhibited greater growth hormone responses to pyridostigmine than the Flinders Resistant Line (FRL) rats. However, there were no significant hypothermic effects of pyridostigmine, suggesting that the sites mediating the growth hormone responses lie outside of the blood-brain barrier. The second aim was to determine whether pyridostigmine had prophylactic effects against the organophosphates chlorpyrifos and diisopropylfluorophosphate regardless of innate cholinergic sensitivity. If anything, the hypothermic responses were greater in the rats pretreated with pyridostigmine, which did, however, protect against diarrhea. These findings indicate that pyridostigmine is not an effective prophylactic against the central effects of organophosphates. Physostigmine, however, was effective in counteracting the hypothermia induced by organophosphates.

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David Overstreet

PI - Signature Date

**Effects of Pyridostigmine in Flinders Line Rats Differing in Cholinergic Sensitivity**


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## INTRODUCTION

In the assessment of risk to individuals exposed to known or potential toxicological agents there needs to be a consideration of the possibility that especially sensitive populations exist. For example, some individuals have reported side effects after taking pyridostigmine to protect them against potential nerve gas exposure and others have not (Jamal, 1998; Shen, 1998). Other individuals have reported increased sensitivity to a variety of chemical agents, usually after a triggering exposure to a specific chemical such as an organophosphate (OP) pesticide (e.g., Miller and Mitzel, 1995). The hypothesis that a genetically based cholinergic supersensitivity might underlie the increased sensitivity of these vulnerable human populations will be addressed in the present communication by describing in detail the features of an animal model with cholinergic supersensitivity that is also more sensitive to a variety of drugs and other chemical agents and that may, therefore, mimic the human condition labeled Multiple Chemical Sensitivity (MCS). In the body of this paper results on the effects of acute pyridostigmine on serum growth hormone levels in this animal model will be presented. In addition, the (in)ability of chronic pyridostigmine to protect these animals against the effects of OPs will be presented and discussed.

### Multiple Chemical Sensitivity

Multiple Chemical Sensitivity (MCS) is a syndrome in which, following acute or repeated exposure to one or more chemicals, most commonly organophosphate pesticides (OPs), individuals become overly sensitive to a wide variety of chemically-unrelated compounds. These can include ethanol, caffeine and other psychotropic drugs (Ashford and Miller, 1989, 1991; Bell et al., 1992; Cullen, 1987; Miller, 1994). The symptoms often reported by MCS patients include fatigue, cognitive difficulties, depression, irritability, headaches, dyspnea,

digestive problems, musculoskeletal pain, and numbness in their extremities. These conditions often overlap those of common medical illnesses such as depression, somatization disorder, chronic fatigue syndrome, fibromyalgia, asthma and others (Gruber et al., 1996). However, a distinguishing feature of MCS is the strong belief of the patients that their symptoms are brought on by common exposures to low levels of volatile organic chemicals such as fragrances, insecticides, traffic exhaust, disinfectants and perfumes (Ashford and Miller, 1991).

An important observation in this field is that MCS patients usually report that other individuals simultaneously exposed to similar amounts of pesticides, e.g., family members, friends, or co-workers, did not develop MCS or even experience transient illness. This observation suggests that a subset or subsets of the people may be more vulnerable to developing MCS. Indeed, some (Black et al., 1990; Simon et al., 1990), but not all (Fiedler et al., 1992) researchers have reported greater rates of depression and somatization disorder predating the "initiating" chemical exposure among persons with MCS as compared to controls. Thus, any model must take into account why only some individuals develop MCS after exposures to pesticides or other chemicals.

The FSL Rat Model

One such model which will be described in the subsequent sections of this paper is the FSL (Flinders Sensitive Line) rat. This rat was developed by selective breeding for increased sensitivity to an OP, so it shares some etiologic similarity to patients with MCS who were exposed to pesticides. The FSL rat model is one with which we have had extensive experience, particularly in research on depressive syndromes (Overstreet, 1993; Overstreet and Janowsky, 1991; Overstreet et al., 1995). Analogies between depressed states and MCS, as well as substance hypersensitivities in FSL rats, first brought our attention to the potential value of this

model for experimental studies of MCS, as recently described (Overstreet et al., 1996). Further, because the FSL rats were selectively bred for increased responses to the organophosphate, DFP, it is possible that they may have some special relevance to Gulf War Illness, commonly reported in individuals exposed to the carbamate, pyridostigmine (Jamal, 1998; Shen, 1998)..

Selective Breeding for OP Differences. The FSL rat model arose from a selective breeding program designed to produce two lines of rats, one with high (FSL) and one with low (Flinders Resistant Line - FRL) sensitivity to the anticholinesterase agent, diisopropylfluorophosphate (DFP) (Overstreet et al., 1979; Russell et al., 1982). The selective breeding program, which was initiated at Flinders University in Adelaide, Australia, utilized three somatic measures of DFP's effects (Overstreet et al., 1979; Russell et al., 1982). A rank-order system was used to give equal weighting to each of the three variables. Rats that had the lowest average ranks were intermated to establish and maintain the line of more sensitive rats (FSL), while rats that had the highest average ranks were intermated to establish and maintain the line of more resistant rats (FRL). Subsequent studies showed that randomly bred Sprague-Dawley rats, from which the lines were originally derived, were not different from the FRL rats. On the other hand, FSL rats were significantly more sensitive to DFP than the other two groups (Overstreet et al., 1979; Russell et al., 1982).

Biochemical Mechanisms. This project was initiated, in part, to develop genetically resistant lines of rats so that the biochemical mechanisms of resistance could be compared with those of tolerance. Early studies ruled out changes in acetylcholinesterase as a mechanism to account for the differential sensitivity of FSL and FRL rats to DFP (Overstreet et al., 1979; Russell and Overstreet, 1987; Sihotang and Overstreet, 1983), just as has been found for tolerance development (See Russell and Overstreet, 1987). Because DFP-tolerant rats were

subsensitive to the effects of muscarinic agonists (e.g., Overstreet et al., 1972, 1973, 1974), the effects of muscarinic agonists on the FSL and FRL rats were examined (Overstreet 1986; Overstreet and Russell, 1982; Overstreet et al., 1986a,b). These studies showed that the FSL rats were more sensitive to pilocarpine, arecoline and oxotremorine than were the FRL rats; this supersensitivity was seen for a variety of responses, including hypothermia, reduced locomotor activity, and suppression of bar-pressing for water reward (Overstreet and Russell, 1982). Thus, FSL rats, developed by selectively breeding for increased sensitivity to DFP, exhibited opposite changes in sensitivity to muscarinic agonists compared to DFP-tolerant rats. Developmental studies indicated that the cholinergic hypersensitivityu exhibited by the FSL rats could be observed at a very young age (Daws and Overstreet, 1999; Daws et al.., 1991), suggesting that it is innate.

Biochemical studies indicated that the FSL rats exhibited greater numbers of muscarinic receptor binding sites in the hippocampus, striatum, and hypothalamus than the FRL rats (Daws and Overstreet, 1999; Overstreet et al., 1984; Pepe et al., 1988), but there were no differences in acetylcholine turnover (Overstreet et al., 1984). Thus, once again, the FSL rats appear to represent the converse of DFP-tolerant rats; having increased numbers of receptors rather than reduced numbers (See Russell and Overstreet, 1987). It appears that both tolerance and acute sensitivity to cholinergic agents is related to postsynaptic cholinergic mechanisms rather than presynaptic. Although in both instances, there have been detectable changes in the muscarinic receptors themselves, there are some findings, such as the increased sensitivity of FSL rats to noncholinergic agents (See Section below), which suggest that post-receptor mechanisms may also contribute.

Behavioral Features of FSL Rats. The FSL and FRL rats differ on a large number of behavioral tasks, as recently summarized in several review papers (Overstreet et al., 1995, 1996, 1998). In this section we will highlight a number of the key differences. The FSL rats have been reported to have lower locomotor activity than the FRL rats under a number of experimental conditions (Bushnell et al., 1995; Overstreet, 1986; Overstreet and Russell, 1982) but not all (Criswell et al., 1994; Rezvani et al., 1994). They are even less active when stressed prior to exposure to the open field (Overstreet, 1986; Overstreet et al., 1989).

Results from several other behavioral paradigms are consistent with the view that depressive-like psychomotor retardation symptoms are more apparent in the FSL rats after exposure to stressors. For example, the FSL rats are impaired in active avoidance paradigms compared to the FRL rats (Overstreet and Measday, 1985; Overstreet et al., 1990a, 1992a). Another stress-oriented paradigm which has provided important information about behavioral differences between FSL and FRL rats is the forced swim test. Upon initial exposure in a cylinder (18-20 cm diameter) of water (25 °C), FSL rats are more immobile than the FRL rats (Caberlotto et al., 1998; Overstreet, 1986; Overstreet et al., 1986a, Pucilowski and Overstreet, 1993; Schiller et al., 1992; Zangen et al., 1997, 1999). This exaggerated immobility of the FSL rats is counteracted by chronic but not acute treatment with antidepressants (Overstreet, 1993; Pucilowski and Overstreet, 1993; Schiller et al., 1992; Zangen et al., 1997). These findings provide further support for the contention that the FSL rat is a useful animal model of depression.

There are also differences in reward-related behaviors between the FSL and FRL rats which are consistent with the proposal that the FSL rats are a model of depression. In operant bar-pressing tasks, the FSL rats bar-pressed at lower rates and had to be maintained at a lower

percentage of their free-feeding body weight and have smaller food pellets (37 vs. 45 mg) in order to keep their motivation sufficiently high to complete the session (Bushnell et al., 1995; Overstreet and Russell, 1982). Despite these differences in reward-related and stress-related behaviors, there appear to be no differences between the FSL and FRL rats in the ability to perform a matching-to-sample task (Bushnell et al., 1995). However, this test was carried out under normal, unstressed conditions, and it is not clear whether similar findings would obtain under stressed conditions. For example, FSL and FRL rats have similar amounts of saccharin consumption under baseline conditions, but the FSL rats exhibit greater decreases after exposure to chronic mild stress (Pucilowski et al., 1993).

The FSL rats also have elevated REM sleep and reduced latency to REM sleep (Shiromani et al., 1988, Benca et al., 1996), as has been reported in human depressives (Benca et al., 1992) Human depressives are also more sensitive to the effects of cholinergic agonists on REM sleep latency (Janowsky et al., 1994), but there are no data in the FSL rats regarding drug effects yet.

In sum, the FSL rats and depressed humans exhibit a large number of behavioral and physiological similarities (See Overstreet, 1993; Overstreet et al., 1995, 1996, for details).

Multiple Chemical Sensitivity in FSL Rats. Clinical observations suggest that MCS may be initiated by acute or chronic exposure to a variety of chemical agents (Miller and Mitzel, 1995). Because the FSL rats were selectively bred to have increased responses to the anticholinesterase agent, DFP, it should not be surprising that they exhibited increased sensitivity to muscarinic agonists (Daws et al., 1991; Overstreet, 1986; Overstreet and Russell, 1982; Overstreet et al., 1992a,b; Schiller et al., 1988). It has also been reported that human depressives are also more sensitive to directly acting muscarinic agonists (Gann et al., 1992;

Gillin et al., 1991) as well as anticholinesterases (Gann et al., 1992; Janowsky and Overstreet, 1995; Nurnberger et al., 1989; O'Keane et al., 1992; Schreiber et al., 1992; Sitaram et al., 1987). A similar increased sensitivity to anticholinesterases has been observed in MCS patients (Cone and Sult, 1992; Miller and Mitzel, 1995; Rosenthal and Cameron, 1991) but there are no published data for MCS patients regarding sensitivity to direct cholinergic agonists. FSL rats are also more sensitive to nicotine, which interacts with nicotinic cholinergic receptors (Schiller and Overstreet, 1993).

The cholinergic system interacts with many other major neurotransmitter systems, including serotonergic, dopaminergic, GABAergic, and noradrenergic. Having animals with clear-cut differences in the cholinergic system afforded us the opportunity to test how the FSL and FRL rats differ in response to drugs interacting with these other neurotransmitter systems. Evidence from various drug challenge studies, in which relatively selective drugs are given to FSL and FRL rats, have revealed a substantial number of differences between the FSL and FRL rats, as summarized in Table 1. FSL rats were found to exhibit a greater degree of hypothermia after a variety of drugs which interact with the serotonin 5-HT1A receptor (Wallis et al., 1988; Overstreet et al., 1992a, 1994). This outcome is consistent with much of the evidence suggesting supersensitive serotonergic mechanisms in depressives (Arango et al., 1990; Arora and Meltzer, 1989; Mikuni et al., 1991), but is not consistent with neuroendocrine studies reporting blunted responses to serotonergic agonists, which suggests serotonergic hyposensitivity (Lesch et al., 1990; Meltzer and Lowy, 1987). There are no data on the effects of selective serotonergic agents in MCS patients, but there is one report of supersensitive responses in individuals with chronic fatigue syndrome, which is related to MCS (Backheit et al., 1992).

To date no evidence has been obtained to indicate any differences in responses to noradrenergic agents in the FSL rats (Overstreet, 1989; Overstreet et al, 1989). In contrast, there are quite a number of differences with regard to dopaminergic agents (Table 1). The FSL rats are supersensitive to the hypothermic (Crocker and Overstreet, 1991) and aggression-promoting (Pucilowski et al., 1991) effects of apomorphine, a mixed D1/D2 agonist, and quinpirole, a selective D2 agonist. On the other hand, the FSL rats were subsensitive to the stereotypy-inducing effects of similar doses of the same compounds and there were no apparent differences in dopamine D2 receptors between FSL and FRL rats (Crocker and Overstreet, 1991). These opposite changes in sensitivity in the various functions might be related to the type of modulation of these functions by the cholinergic and dopaminergic systems. Stimulation of both cholinergic and dopaminergic systems promotes hypothermic and aggressive responses (Cox et al., 1980; Pucilowski, 1987; Ray et al., 1989), but cholinergic stimulation reduces activity and stereotypy, thereby opposing the effects of dopaminergic stimulation (Fibiger et al., 1970; Klemm, 1989).

The FSL and FRL rats are differentially sensitive to the effects of several pharmacological agents which have modulatory roles at the GABA-A receptor, as summarized in Table 1. However, as with the case of dopamine agonists, the differential effects are observed only for some actions of the drugs, not for all. For example, the hypothermic effects of ethanol are significant higher in the FSL rats compared to the FRL rats, but the sedative effects are similar (Overstreet et al., 1990b). Similarly, the behavioral suppressant effects of diazepam are significantly greater in the FSL rats (Pepe et al., 1988), but its anxiolytic effects in the two lines are comparable (Schiller et al., 1991). The fact that these two commonly abused psychotropic drugs both modulate GABA function at the GABA-A receptor suggests that there might be

differences in GABA-A receptor subtype composition between the two lines, but there is not biochemical evidence for such differences as yet. Furthermore, despite differences in sensitivity to the hypothermic effects of ethanol, the FSL and FRL rats do not differ in their rates of voluntary ethanol consumption (Overstreet et al., 1992a).

In summary, it appears that the FSL rat is more sensitive to a variety of chemical agents in addition to the OP anticholinesterase agent for which they were selectively bred. In this regard, the FSL rat is somewhat analogous to MCS patients who have become more sensitive to a range of agents following exposure to OP anticholinesterases. The extent of the similarity between the FSL rats and MCS patients, on one hand, and human depressives and MCS patients, on the other, has been more extensively evaluated in recent reviews (Overstreet et al., 1996, 1997a).

Acute Effects of Pyridostigmine

Pyridostigmine bromide is a quaternary carbamate anticholinesterase agent which has been used routinely in the treatment of myasthenia gravis (Keesey, 1998; Taylor, 1996). It was prescribed to Persian Gulf War participants as a prophylactic against the possible exposure to nerve agents (Keeler et al., 1991). A subset of these individuals have reported very various problems, but it is not yet clear whether the problems are related to their exposure to pyridostigmine, to other agents during the Gulf War, or to stress (Jamal, 1998; Shen, 1998). The present proposal addresses the hypothesis that the individuals developing these problems may have had a genetic cholinergic supersensitivity, undetectable under normal conditions, which made them more sensitive to pyridostigmine and/or other agents to which they were exposed. Because the FSL and FRL rats were genetically selected to respond differently to cholinergic agonists, they are ideal animals to test this hypothesis. It was predicted that the

cholinergically supersensitive FSL rats would be more sensitive to the effects of pyridostigmine than the FRL rats or an outbred Sprague-Dawley strain of rats. The serum levels of growth hormone were selected as one variable to assess because there is evidence that pyridostigmine produces exaggerated elevations of this hormone in several human populations with abnormalities (Chaudhury et al., 1997; Ghigo et al., 1993; Lucey et al., 1993; O'Keane et al., 1992, 1994; Cooney et al., 1997a,b). Telemetrically monitored core body temperature and general activity were selected as additional variables which could be measured reliably without influencing growth hormone levels and which might also be affected by pyridostigmine.

Chronic Effects of Pyridostigmine.

Pyridostigmine was given chronically to troops which had been assigned to duty in the Gulf War in the hope that it would protect them against the consequences of possible exposure to nerve agents which, like pyridostigmine, inhibit cholinesterases. Because it had been widely used in the treatment of myasthenia gravis (Taylor, 1996) without incident and had been given chronically to a group of human volunteers maintained under high ambient temperatures (Wenger et al., 1993), it was assumed that pyridostigmine itself would not have any negative consequences. However, whether it could act as a prophylactic against the effects of nerve agents had not been adequately tested at the time this proposal was submitted, although there had been reports of its usefulness in conjunction with other prophylactics such as oximes and anticholinergics (e.g., Koplovitz and Stewart, 1994).

The basic thesis of our proposal is that there will be individual differences in sensitivity to pyridostigmine and to nerve agents and that, consequently, pyridostigmine may not be able to protect all individuals from the effects of exposure to nerve agents. To address this question, it was proposed to use both sexes of the FSL and FRL rats, differentially sensitive to cholinergic

agents, and SD rats which had been chronically treated with saline or pyridostigmine for two weeks. The animals would then be challenged with the commonly used pesticide, chlorpyrifos (CPF), or the commonly used experimental anticholinesterase agent, diisopropylfluorophosphate (DFP), and core temperature and activity recorded.

## BODY

Methods

Animals. The FSL and FRL rats were selected from breeding colonies maintained at the University of North Carolina at Chapel Hill and randomly bred Sprague-Dawley (SD) rats (from which the FSL and FRL rats were originally derived) were obtained from Harlan Sprague-Dawley (Indianapolis, IN) to act as a reference group. Both males and females were used. The SD rats were included in the research design in order to determine whether both FSL and FRL rats are different from this randomly bred population. Until surgery they were maintained in groups of 3-5 in polycarbonate cages under conditions of constant temperature and humidity and a reversed light:dark cycle (lights off from 1000-2200).

Surgery. Recording of locomotor activity and core body temperature in freely moving rats was accomplished by the implantation of a transmitter weighing 7.0 g (Model TA-11ETA-F40-L20). This transmitter had temperature- and motion-sensitive elements and when actuated by passing a magnet along the rat's abdomen, transmitted information to a computer where it was stored using Data Quest IV software (Data Sciences, Inc., St. Paul, MN).

At about 70 days of age the rats were injected i.p. with sodium pentobarbital (35 mg/kg) to induce anesthesia for implanting the telemetry transmitters, which provided continuous monitoring of core body temperature and general activity. The fur over the ventral abdominal area was clipped and a 3-cm longitudinal incision was made along the midline about 1 cm below

the sternum. The radiotransmitter was inserted into the abdominal cavity and sutured to the peritoneal wall with 4-0 silk thread. After testing the transmitter with an AM receiver, the skin was closed. The rats were placed in single polypropylene cages after surgery and were closely monitored until they were active.

Acute Pyridostigmine Procedures. After a one week period to allow full recovery (Rezvani et al., 1994), the FSL, FRL and SD rats were adapted to the home cages for at least 24 hr and then injected s.c. with a mixture of peripherally acting methyl atropine (MA, 2.0 mg/kg) and oxotremorine (OXO, 0.2 mg/kg) to determine hypothermic responses. This treatment was given to insure that each group of rats were either sensitive (FSL) or resistant (FRL) to a well characterized cholinergic agonist. This information is necessary to interpret the hypothermic responses to pyridostigmine.

Approximately three days after the MA/OXO challenge, the rats were given pyridostigmine (PYR) bromide by gavage. The design called for four groups (vehicle and 4, 12, 36 mg/kg), with ten rats per group. The animals were run in squads of 10 rats, the capacity of the computer, in a counterbalanced order. The average temperatures and general activity counts recorded during the hour preceding the gavage and those recorded at approximately 30 min after the injection were used in statistical analyses.

The rats were sacrificed by decapitation exactly 30 min after the oral administration of pyridostigmine, any signs of diarrhea were noted, and blood was collected into centrifuge tubes. The tubes were centrifuged and the plasma was collected and stored at -20 °C for later determination of growth hormone levels, using a kit obtained by NIDDK.

Chronic Pyridostigmine Procedures. The intermediate dose of 12 mg/kg pyridostigmine was selected as the chronic dose because it produced a significant elevation of growth in every

group (Fig. 1) ; it was gavaged in a volume of 3 ml/kg and the controls received an equivalent volume of isotonic saline orally. Some variations of the above procedures were necessary due to the requirement of a 14-day chronic treatment period with pyridostigmine or saline. Chronic treatment was initiated approximately 1-2 days prior to surgery to implant the transmitter. Surgery was performed in the afternoon, approximately 4-6 hr after the daily treatment with pyridostigmine and proceeded without incident. After a one week period of recovery, the rats were placed on the receivers for the monitoring of temperature and activity baselines for at least 48 hr prior to the challenge with CPF or DFP.

Exactly 30 min after the 14th treatment with pyridostigmine, the rats received one of three challenge treatments: Saline (3 ml/kg orally), CPF (60 mg/kg in 3 ml/kg orally), or DFP (1 mg/kg intramuscularly). Temperature and activity were monitored for the next two hr and then the rats were sacrificed by decapitation. The blood was stored for the later determination of cholinesterase activity and possible growth hormone levels and the brains were stored for the later determination of cholinesterase activity and muscarinic receptor binding. Only the physiological data will be communicated in this report. The assays for the biochemical measures have not been completed as yet.

There was also a variation in the timing of the MA/OXO challenge as the experiments progressed. Initially, the challenge was conducted two days prior to the start of the chronic treatment phase, using a Physiotemp telethermometer and temperature probe. After the recording of baseline temperatures, the rats were given sc injections of the MA/OXO (2/0.2 mg/kg) mixture and core body temperatures were recorded at 30, 60 and 90 min after the injections. These recordings provided information about the sensitivity of the cholinergic system in the various groups prior to the start of chronic pyridostigmine or saline treatment.

When it became apparent that pyridostigmine was altering the hypothermic sensitivity to CPF, the timing of the MA/OXO challenge was changed to nine days after the initiation of the chronic treatment period (approximately one week after the implantation of the transmitters). Temperature was now monitored telemetrically and 48 hr of baseline and the complete time course of oxotremorine-induced hypothermia were recorded. This procedure was adopted so that the potential effects of chronic pyridostigmine on a cholinergic drug which is not dependent upon cholinesterase inhibition for its effects. Oxotremorine interacts directly with central cholinergic receptors (since its peripheral effects were blocked by MA) to induce its hypothermic effects.

Results and Discussion

Acute Pyridostigmine. The effects of acute pyridostigmine or saline treatment on activity and temperature were described in last year's annual report. Table 2 from that report is included here to emphasize the lack of strain and gender differences in the changes in temperature produced by either compound (See Table 2). For the 4 mg/kg dose only, the female rats exhibited larger increases in temperature than their male counterparts. The serum growth hormone assays from these animals have now been completed and are included in this report (Fig. 1).

The values for growth hormone levels at 30 min after the administration of pyridostigmine are illustrated in Figure 1. The data were initially analyzed for gender differences and, since none were found, the results for males and females were combined for each line. In each line, pyridostigmine produced an elevation at the intermediate doses, with 12 mg/kg being significantly higher than saline in each line. However, both the FSL and the SD rats exhibited

greater elevations of growth hormone than the FRL rats at the 4 mg/kg dose of pyridostigmine, suggesting greater cholinergic sensitivity in these lines of rats.

When considered in conjunction with the negative data on temperature and activity presented in our previous annual report, these results suggest that the site(s) with which pyridostigmine interacts to induce increases in growth hormone may be located outside of the central nervous system or the blood-brain barrier. Irrespective of this conclusion, it is also clear that the FSL rats exhibited a greater growth hormone elevation to pyridostigmine than did the FRL rats. Such a finding would be consistent with the suggestion that the FSL rat may be a genetic animal model of depression, because depressed humans are also more sensitive to pyridostigmine-induced changes in growth hormone (O'Keane et al., 1992; Cooney et al., 1997b).

The SD rats appeared to be as sensitive to the effects as pyridostigmine on growth hormone as the FSL rats (Fig. 1), but were intermediate in their sensitivity to oxotremorne (Fig. 2). These SD rats were obtained from Harlan and there has been other evidence that this substrain of SD may be very different from another substrain of SD rats obtained from Holtzman. For example, it has been recently reported that the SD/Har rats are more sensitive to the hypothermic effects of 8-OH-DPAT, a selective serotonin1A (5-HT1A) receptor agonist, than are the SD/Hol rats (Balcells-Olivero et al., 1997, 1998). We have also reported that the FSL rats are similarly more sensitive to the hypothermic effects of 8-OH-DPAT compared to the FRL rats (Overstreet et al., 1994). Because it is likely that the cholinergic and serotonergic systems interact (Overstreet et al., 1998b), it is possible that the SD/Har rats may be more sensitive than the SD/Hol rats to cholinergic agonists as well as 5-HT1A receptor agonists.

Another recent report has also emphasized the differences in drug responses between Sprague-Dawley rats obtained form different suppliers (Trujillo et al., 1998).

In conclusion, it should be stressed that the FSL rats exhibited a supersensitive growth hormone response to pyridostigmine compared to the FRL rats. Since there were no changes in temperature and activity in these same rats, these findings are evidence for the growth hormone changes probably being mediated by sites outside of the blood-brain barrier. Thus, the cholinergic supersensitivity exhibited by the FSL rats can be observed at sites outside of the brain. This conclusion is consistent with other recent findings in these rats indicating increased sensitivity of the FSL rats to cholinergic drugs applied to the small intestine (Djuric et al., 1995) and the airways (Djuric et al., 1998).

Chronic Pyridostigmine. The results of the MA/OXO challenges on core body temperature prior to the beginning of chronic treatment are summarized in Figure 2. The FSL rats are clearly more sensitive to the hypothermic effects of MA/OXO and the FRL rats are resistant, both in reference to the FSL rats and the randomly bred SD rats. Note also that the female rats are more sensitive. These data, therefore, confirm the differences seen previously using telemetrically monitored temperature (Overstreet et al., 1997b, 1998a).

A wealth of physiological data has been collected in this project. Only the data on temperature will be summarized here. To illustrate the changes in temperature which occurred during the various treatments, we have decided to present the data as a series of graphs containing the last 4.5 hr prior to the last chronic treatment, the 30 min following this last chronic treatment and the 2 hr following the challenge treatment. Manipulations of these basic data have then been conducted to provide statistical analyses of the respective groups.

The first set of figures illustrates that a saline challenge in the rats chronically treated with pyridostigmine or saline produced relatively few effects, as might be expected (Fig. 3A-F). However, there are distinct trends in the data for the some of rats chronically treated with pyridostigmine to have elevated or reduced temperatures relative to the saline-treated controls (See Fig. 3). As a consequence of this finding, the effects of challenge drugs were expressed as changes from the 30-min temperatures just prior to their administration.

The second set of figures illustrates the hypothermic effects of CPF in rats chronically treated with pyridostigmine or saline. In five out of six of the groups the rats that had been chronically pretreated with pyridostigmine exhibited a more rapid decrease in temperature after CPF challenge (See Figure 4). As illustrated in Figure 5, DFP also produced a more rapid decrease in temperature in all of the rats chronically pretreated with pyridostigmine. These effects were quite striking. As indicated in Figures 4 and 5, the groups chronically pretreated with pyridostigmine exhibited more rapid decreases in temperature than the groups chronically pretreated with saline. To evaluate these differences, the average temperatures of the various treatment groups at 1 and 2 hr after the acute challenge treatments of DFP and CPF (1.5 and 2.5 hr after the last pyridostigmine or saline treatment) were compiled in tabular form and analyzed by two-way Analysis of Variance, with strain and pretreatment as the two main factors. These findings are summarized in Tables 3 & 4. Pretreatment effects were significant at 1 but not a 2 hr, while strain differences were significant at both time points. In all cases, the FRL rats were the most resistant, while the FSL rats were the most sensitive to DFP but equally as sensitive to CPF as the SD rats.

The greater effects of both CPF and DFP in the pyridostigmine-pretreated rats could be explained by a pharmacokinetic mechanism. Because pyridostigmine is attached to

cholinesterase molecules in the periphery, there are fewer binding sites for CPF and DFP. Therefore, more of these agents should penetrate the brain and one could therefore expect a more rapid rate of decline in core body temperature, as seen in Figs. 4 & 5. If this hypothesis is correct, then the brain cholinesterase activity may be lower in the rats that had been pretreated with pyridostigmine and acutely challenged with CPF or DFP. The results of these cholinesterase assays are not available yet, but will be communicated at a later date.

Another approach to account for the differences in sensitivity in pyridostigmine- and saline-treated rats is to introduce a challenge to an agent whose effects are not dependent on cholinesterase inhibition. As indicated in Figure 6, it appears that chronic pyridostigmine treatment also sensitized some animals to the hypothermic effects of OXO, a directly acting cholinergic receptor agonist. However, the pattern of temperature changes is different for OXO than for CPF or DFP. For all three drugs the peak change in temperature was fairly similar for rats pretreated with either pyridostigmine or saline. For OXO, there was a prolonged hypothermia in the pyridostigmine-treated FSL groups (Fig. 6B & 6E), while it was shorter in the SD males (Fig. 6D). In contrast, for CPF and DFP, there was a more rapid decline in body temperature in almost all groups (Fig. 4 & 5).

Assessment of Diarrhea. Diarrhea is a frequent symptom in animals exposed to anticholinesterase agents and is probably a reasonable index of peripheral cholinergic overstimulation. Evidence of diarrhea was observed at the time of sacrifice two hr after administration of the CPF, DFP or saline challenges and 2.5 hr after the last treatment with pyridostigmine or saline. There were no signs of diarrhea in the rats challenged with saline, confirming the low incidence of diarrhea by this oral dose of pyridostigmine reported in our last annual report. CPF, which was orally administered at a intermediate dose of 60 mg/kg

(Nostrandt et al., 1997) also produced relatively little diarrhea, with only two FSL male rats (one pretreated with pyridostigmine, one pretreated with saline) showing signs of diarrhea. The incidence of diarrhea was higher following the sc administration of 1 mg/kg DFP. Across all groups, a total of 2 out of 55 rats pretreated with pyridostigmine showed signs of diarrhea after DFP challenge, while 16 out of 58 rats pretreated with saline were similarly affected (chi square = 11.59, $p < 0.001$). Almost all of the animals exhibiting diarrhea were either FSL or SD rats, suggesting increased peripheral cholinergic sensitivity in these groups, a conclusion which has been reinforced by other recent studies (Djuric et al., 1995; 1998). These findings are also suggestive of the possibility that pyridostigmine may offer protection against a key peripheral cholinergic symptom induced by DFP. These findings are consistent with other reports of the effectiveness of pyridostigmine in protecting against the peripheral and/or lethal effects of organophosphate nerve agents (e.g. French et al., 1979; Dirnhuber et al., 1979; Xia et al., 1981; Walday et al., 1993).

Assessment of Physostigmine's Protective Effects. Several investigators have reported that physostigmine, a centrally acting carbamate anticholinesterase agent, is more effective than pyridostigmine in protecting against the effects of organophosphates (Deshpande et al., 1986; Phlippens et al., 1998; Solana et al., 1990). Therefore, an experiment was conducted to assess the acute protective effect of physostigmine against DFP. Surgery was conducted to implant the transmitters. After one week of recovery the rats were placed into the telemetry chambers for one day to allow adaptation. Then rats were given 4 mg/kg physostigmine or an equivalent volume of saline by gavage. This was followed 30 min later by the DFP (1 mg/kg, i.m.) challenge. Temperature was recorded for a further two hours. It can be seen that the FSL rats initially exhibit a robust hypothermic response to physostigmine itself, whereas the FRL rats do

not. Nevertheless, the rats pretreated with saline exhibited greater decreases in temperature at two hr than the rats pretreated with physostigmine, regardless of gender or line (Fig.7). The shape of the graphs in Fig. 7 is quite distinct from that shown in Fig. 5 for pyridostigmine. Therefore, physostigmine is more effective than pyridostigmine in protecting against the hypothermic effects of DFP.

Table 1

Multiple Chemical Sensitivity in FSL Rats

Drug Classes to which FSL rats are more sensitive than FRL rats

| Drug Class | Compound | Responses |
|---|---|---|
| Anticholinesterase | DFP | Temperature/drinking |
| Anticholinesterase | Physostigmine | Temperature/activity |
| Muscarinic Agonist | Oxotremorine | Temperature/activity |
| Muscarinic Agonist | Pilocarpine | Temperature/activity |
| Muscarinic Agonist | Arecoline | Temperature/activity |
| Nicotinic Agonist | Nicotine | Temperature/activity |
| Dopamine D1/2 Agonist | Apomorphine | Temperature |
| Dopamine D2 Agonist | Quinpirole | Temperature |
| Dopamine D2 Antagonist | Raclopride | Catalepsy |
| 5-HT-1B Agonist | mCPP | Temperature/activity |
| 5-HT-1A Agonist | 8-OH-DPAT | Temperature |
| 5-HT-1A Agonist | Buspirone | Temperature |
| Benzodiazepine Agonist | Diazepam | Temperature/activity |
| Multiple (GABA, 5-HT) | Ethanol | Temperature |

Table 2

Change in Core Temperature after Oral Administration of Saline or Pyridostigmine in FSL, FRL and SD Rats

| Line/Sex | Dose of Pyridostigmine (mg/kg) | | | |
|---|---|---|---|---|
| | 0.0 | 4.0 | 12.0 | 36.0 |
| SD-male | +0.4±0.1 | +0.6±0.1[b] | +0.2±0.1 | 0.0±0.2 |
| SD-female | +0.5±0.1 | +1.0±0.1[a] | +0.5±0.1 | -0.2±0.2 |
| FSL-male | +0.3±0.3 | +0.3±0.2[c] | +0.9±0.4 | -0.2±0.2 |
| FSL-female | +0.4±0.2 | +0.7±0.1[b] | +0.8±0.2 | +0.1±0.2 |
| FRL-male | +0.3±0.2 | +0.2±0.1[c] | +0.3±0.2 | +0.1±0.2 |
| FRL-female | +0.3±0.2 | +0.4±0.2[b,c] | +0.8±0.1 | +0.2±0.1 |
| One-Way ANOVA | 0.50 | 5.17** | 2.55 | 0.91 |

**Significant differences, $p < 0.01$

Groups with different letters are significantly different, $p < 0.05$, Tukey's test.

Table 3

Hypothermic Effects one hour after DFP or CPF Treatment in Rats Chronically Pretreated with Saline or Pyridistigmine

| STRAIN/SEX | CPF | | DFP | |
|---|---|---|---|---|
| | SAL | PYR | SAL | PYR |
| SD-F | 36.90±0.18[a] | 36.07±0.08[a] | 37.27±0.25[a] | 36.95±0.09[a] |
| FSL-F | 37.13±0.11[a] | 36.47±0.09[b] | 36.29±0.34[b] | 35.07±0.19[b] |
| FRL-F | 37.85±0.04[b] | 37.53±0.04[c] | 37.87±0.11[c] | 37.32±0.07[c] |
| SD-M | 37.18±0.05[a] | 36.93±0.06[d] | 37.28±0.15[a] | 36.62±0.15[a] |
| FSL-M | 36.70±0.12[c] | 36.83±0.09[d] | 36.95±0.19[b] | 35.78±0.23[b] |
| FRL-M | 37.17±0.03[a] | 37.00±0.04[d] | 37.70±0.07[c] | 37.20±0.08[c] |
| F (Treatment) = | 14.52, $p < 0.01$ | | 11.22, $p < 0.01$ | |
| F (Strain/Sex) | 81.47, $p < 0.001$ | | 109.76, $p < 0.001$ | |

Groups with different letters are significantly different, $p < 0.05$, Tukey's test.

Table 4

Hypothermic Effects two hours after DFP or CPF Treatment in Rats Chronically Pretreated with Saline or Pyridistigmine

| STRAIN/SEX | CPF | | DFP | |
|---|---|---|---|---|
| | SAL | PYR | SAL | PYR |
| SD-F | $35.20\pm0.08^{a}$ | $35.34\pm0.06^{a}$ | $35.43\pm0.09^{a}$ | $36.10\pm0.09^{a}$ |
| FSL-F | $36.07\pm0.07^{b}$ | $35.64\pm0.06^{a}$ | $34.50\pm0.11^{b}$ | $34.53\pm0.06^{b}$ |
| FRL-F | $37.41\pm0.10^{c}$ | $37.47\pm0.06^{b}$ | $37.26\pm0.06^{c}$ | $37.16\pm0.05^{c}$ |
| SD-M | $36.10\pm0.12^{b}$ | $35.78\pm0.12^{a}$ | $36.07+0.07^{d}$ | $35.73\pm0.10^{a}$ |
| FSL-M | $35.64\pm0.11^{a,b}$ | $35.94\pm0.07^{a}$ | $35.23\pm0.12^{a}$ | $34.56\pm0.04^{b}$ |
| FRL-M | $37.03\pm0.06^{c}$ | $36.87\pm0.08^{c}$ | $36.94\pm0.06^{c}$ | $36.69\pm0.10^{d}$ |
| F(treatment) = | 0.45, NS | | 0.75, NS | |
| F (strain/sex) | 481.36, $p < 0.001$ | | 667.5, $p < 0.001$ | |

Groups with different letters are significantly different, $p < 0.05$, Tukey's test.

Figure Captions

Figure 1. Dose-Dependent Effects of Pyridostigmine on Serum Growth Hormone Levels in FSL, FRL and SD Rats. Rats were treated with pyridostigmine or saline by gavage 30 min prior to sacrifice by decapitation. Blood was collected in heparinized tubes and stored frozen at -20 °C until assayed by a kit from NIDDK. Values represent the mean values for 9-15 rats. *Significantly different from saline treatment.

Figure 2. Strain and Gender-Dependent Effects of Oxotremorine in FSL, FRL and SD Rats. After the recording of baseline temperatures, rats were injected sc with a mixture of 2 mg/kg methyl atropine nitrate and 0.2 mg/kg oxotremorine sesquifumarate. The scores represent the mean decrease from baseline temperature (°C) for 25-30 rats at 60 min after the injection. Temperatures were recorded by a Bailey's telethermometer. Different letters indicate that the groups are significantly different from each other according to Tukey's tests.

Figure 3A-F. Changes in Telemetrically Monitored Temperature in FSL, FRL and SD Rats following Chronic Saline or Pyridostigmine Treatment and Acute Saline Challenges. Rats were chronically treated by gavage with pyridostigmine (12 mg/kg) or saline for 14 days. The saline challenge (gavage or intramuscular) was given 30 min after the $14^{th}$ treatment and temperature was monitored for a further 2 hr. The data represent the mean temperatures for 8-10 animals. Standard errors are omitted for clarity.

Figure 4A-F. Changes in Telemetrically Monitored Temperature in FSL, FRL and SD Rats following Chronic Saline or Pyridostigmine Treatment and Acute Chlorpyrifos (CPF) Challenges. Rats were chronically treated by gavage with pyridostigmine (12 mg/kg) or saline for 14 days. The CPF challenge (60 mg/kg by gavage) was given 30 min after the $14^{th}$ treatment

and temperature was monitored for a further 2 hr. The data represent the mean temperatures for 8-10 animals. Standard errors are omitted for clarity.

Figure 5A-F. Changes in Telemetrically Monitored Temperature in FSL, FRL and SD Rats following Chronic Saline or Pyridostigmine Treatment and Acute Diisopropylfluorophosphate (DFP) Challenges. Rats were chronically treated by gavage with pyridostigmine (12 mg/kg) or saline for 14 days. The DFP challenge (1 mg/kg, s.c.) was given 30 min after the 14$^{th}$ treatment and temperature was monitored for a further 2 hr. The data represent the mean temperatures for 8-10 animals. Standard errors are omitted for clarity.

Figure 6A-F. Changes in Telemetrically Monitored Temperature in FSL, FRL and SD Rats following Chronic Saline or Pyridostigmine Treatment and Acute Oxotremorine (OXO) Challenges. Rats were chronically treated by gavage with pyridostigmine (12 mg/kg) or saline for 10 days. The OXO challenge (0.2 mg/kg with 2 mg/kg methyl atropine, s.c.) was given 30 min after the 10$^{th}$ treatment and temperature was monitored for a further 6 hr. The data represent the mean temperatures for 8-10 animals. Standard errors are omitted for clarity.

Figure 7. Changes in Telemetrically Monitored Temperature in FSL and FRL rats following Acute Pretreatment with Saline or Pyridostigmine and Acute DFP challenges. Rats were pretreated with physostigmine (4 mg/kg) by gavage. The DFP challenge (1.0 mg/kg, i.m.) was given 30 min after the physostigmine pretreatment. Temperature was monitored for two hours after the DFP treatment. The data represent the mean temperatures for 8-10 animals. Standard errors are omitted for clarity.

FIGURE 1

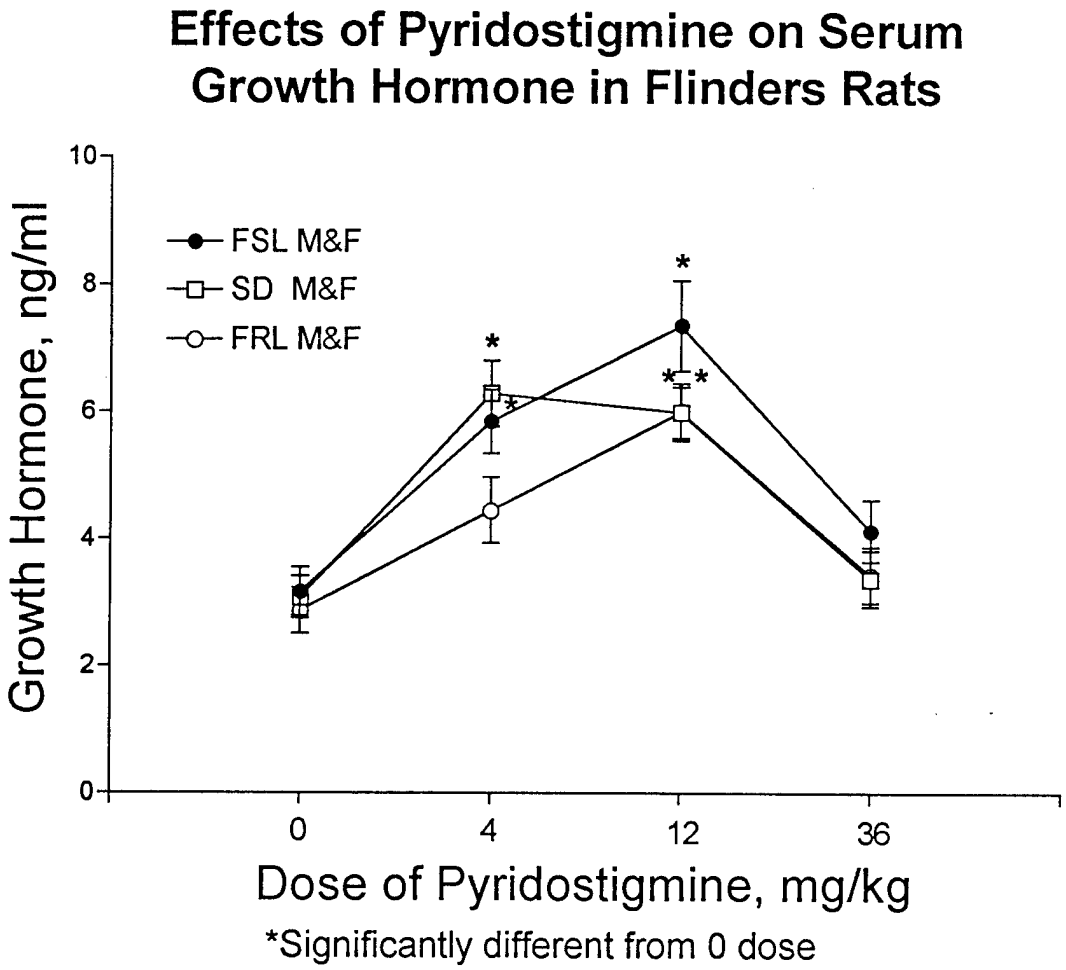

Effects of Pyridostigmine on Serum Growth Hormone in Flinders Rats
Growth Hormone, ng/ml
10
8
6
4
2
0
FSL M&F
SD M&F
FRL M&F
*
*
*
*
*
0
4
12
36
Dose of Pyridostigmine, mg/kg
*Significantly different from 0 dose

FIGURE 2

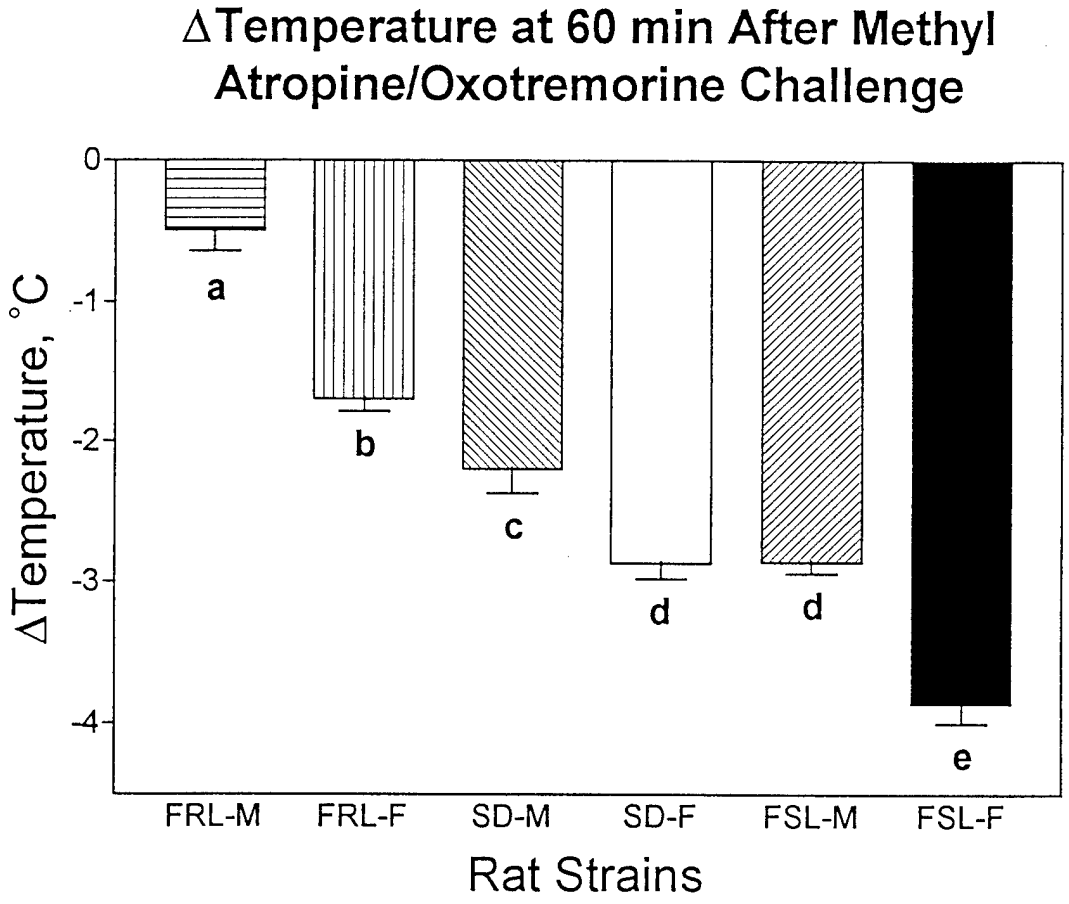
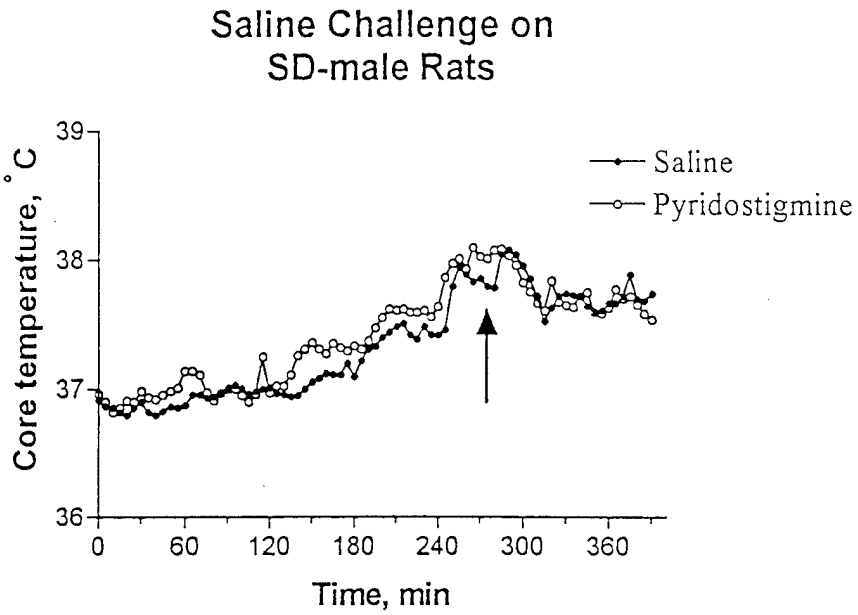
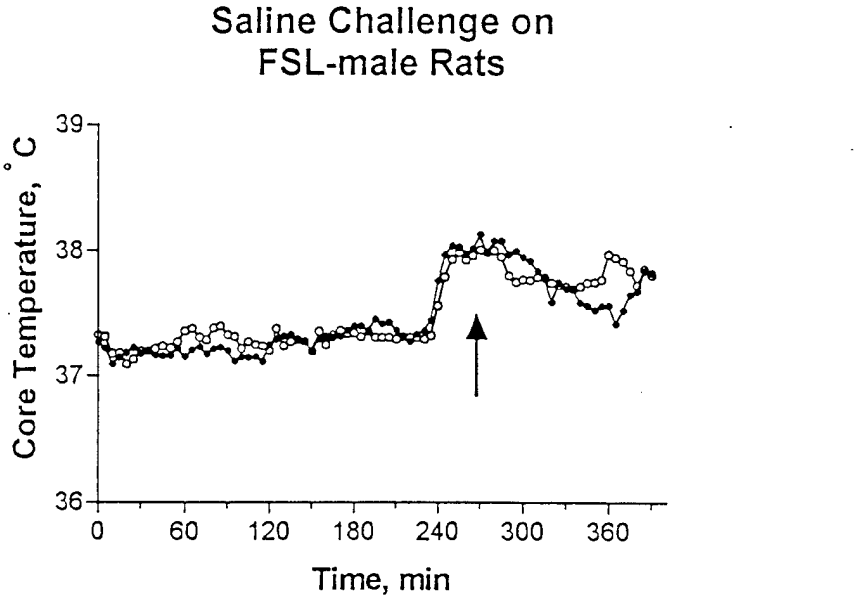

ΔTemperature at 60 min After Methyl Atropine/Oxotremorine Challenge
ΔTemperature, °C
0
-1
-2
-3
-4
a
b
c
d
d
e
FRL-M
FRL-F
SD-M
SD-F
FSL-M
FSL-F
Rat Strains

FIGURE 3

A


Saline Challenge on
SD-male Rats
Saline
Pyridostigmine
Core temperature, °C
39
38
37
36
0
60
120
180
240
300
360
Time, min

B


Saline Challenge on
FSL-male Rats
Core Temperature, °C
39
38
37
36
0
60
120
180
240
300
360
Time, min

C

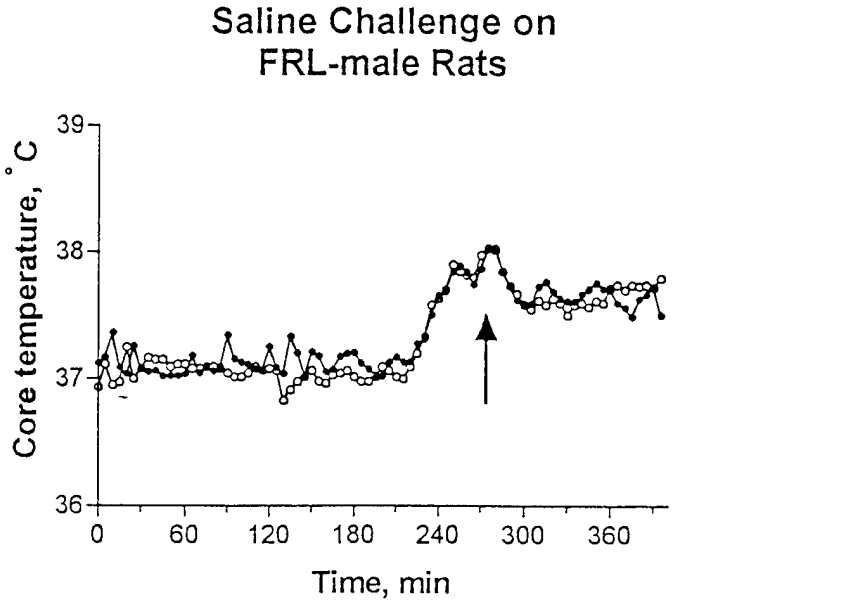
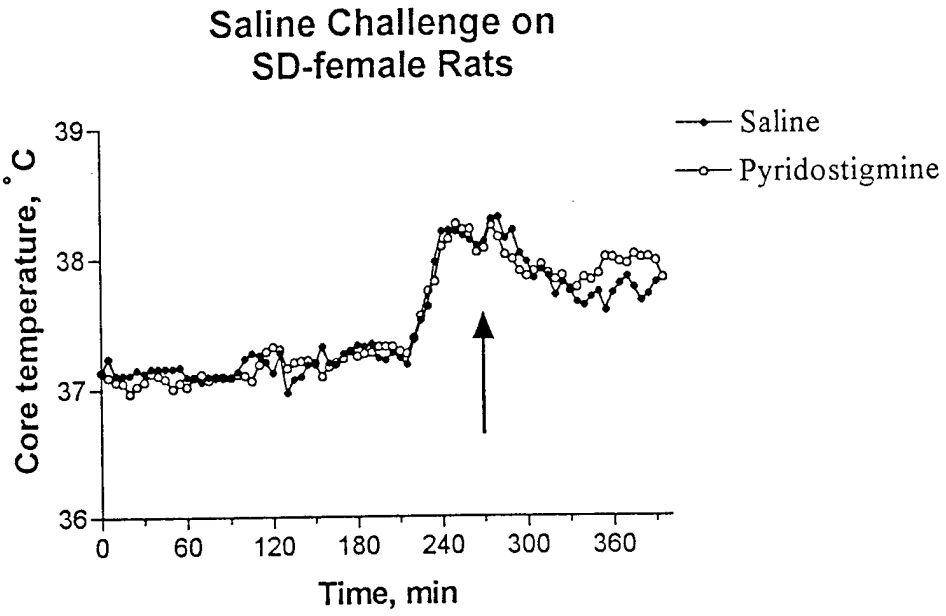
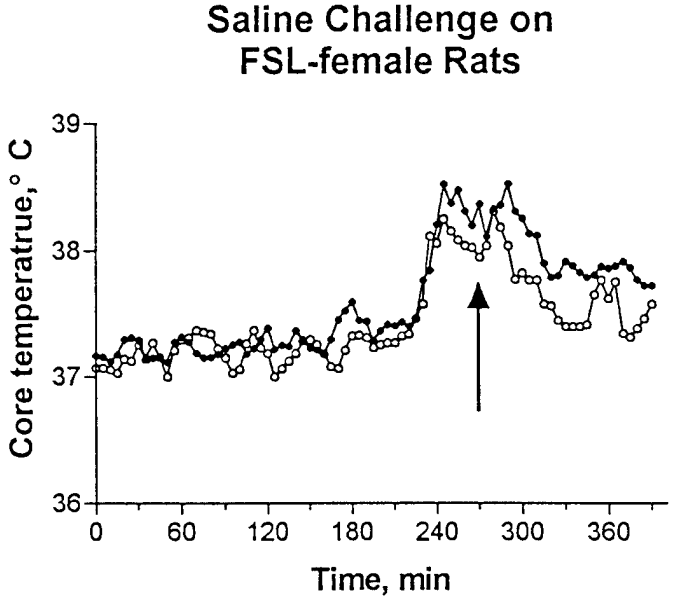

Saline Challenge on
FRL-male Rats
Core temperature, °C
39
38
37
36
0
60
120
180
240
300
360
Time, min

FIGURE 3

D


Saline Challenge on
SD-female Rats
Saline
Pyridostigmine
Core temperature, °C
39
38
37
36
0
60
120
180
240
300
360
Time, min

E


Saline Challenge on
FSL-female Rats
Core temperatrue, ° C
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60
120
180
240
300
360
Time, min

F

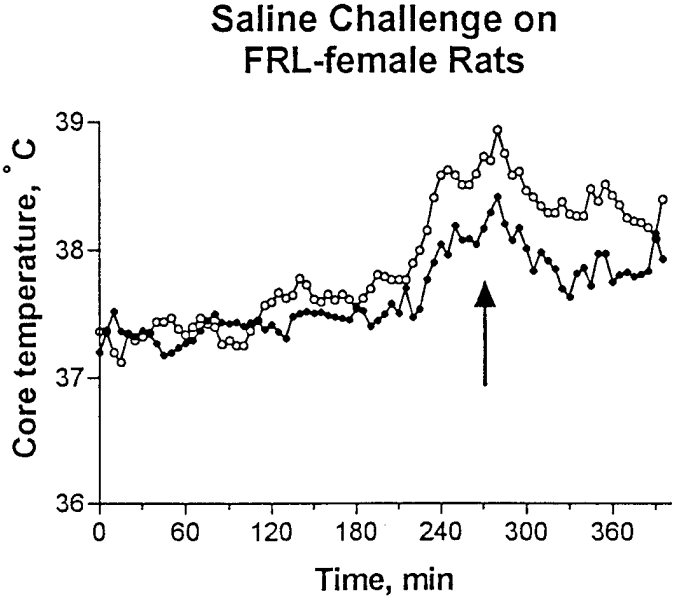
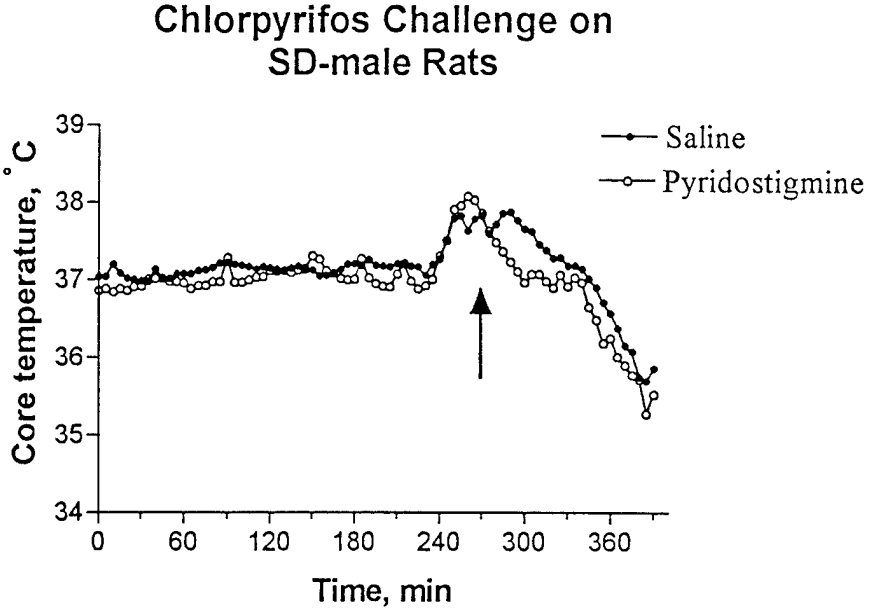

Saline Challenge on
FRL-female Rats
Core temperature, °C
39
38
37
36
0
60
120
180
240
300
360
Time, min

FIGURE 4

A


Chlorpyrifos Challenge on
SD-male Rats
Saline
Pyridostigmine
Core temperature, °C
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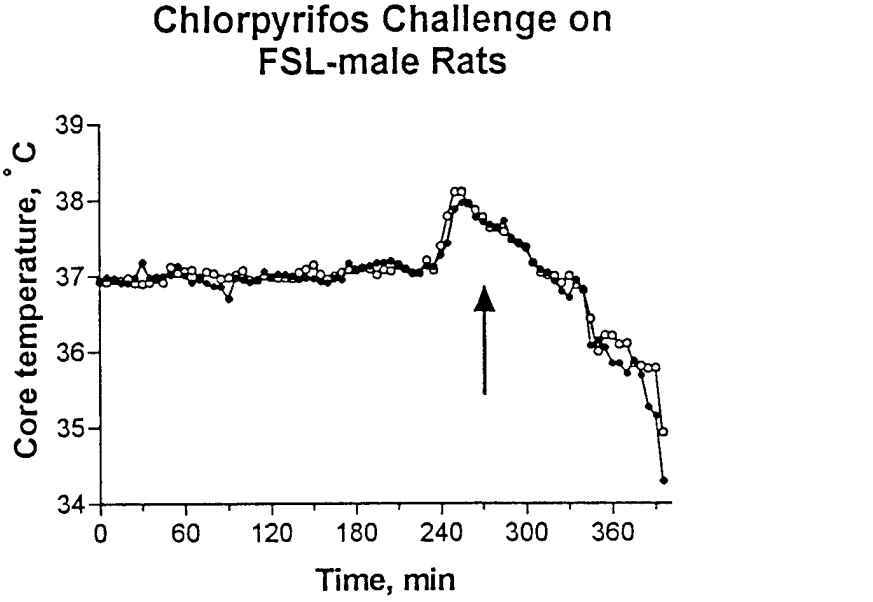
38
37
36
35
34
0
60
120
180
240
300
360
Time, min

B


Chlorpyrifos Challenge on
FSL-male Rats
Core temperature, °C
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38
37
36
35
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120
180
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360
Time, min

C

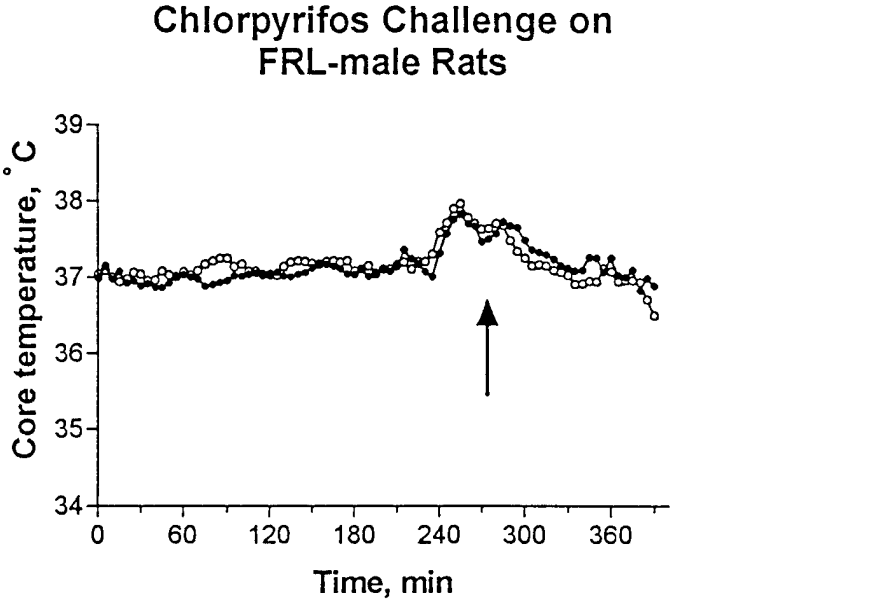
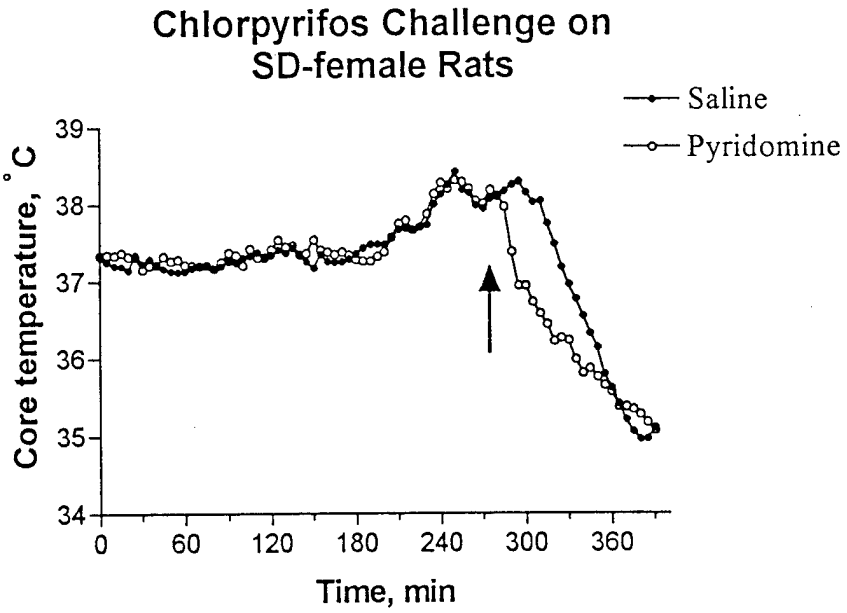

Chlorpyrifos Challenge on
FRL-male Rats
Core temperature, °C
39
38
37
36
35
34
0
60
120
180
240
300
360
Time, min

FIGURE 4

D


Chlorpyrifos Challenge on
SD-female Rats
Saline
Pyridomine
Core temperature, °C
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36
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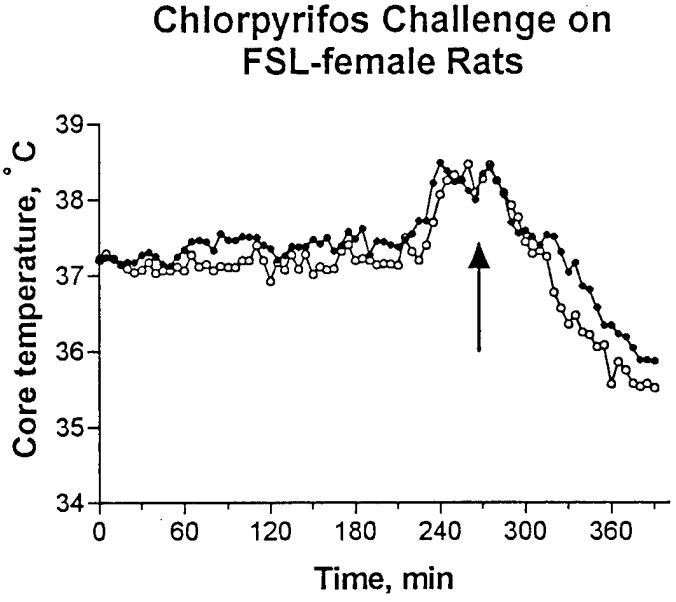
60
120
180
240
300
360
Time, min

E


Chlorpyrifos Challenge on
FSL-female Rats
Core temperature, °C
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34
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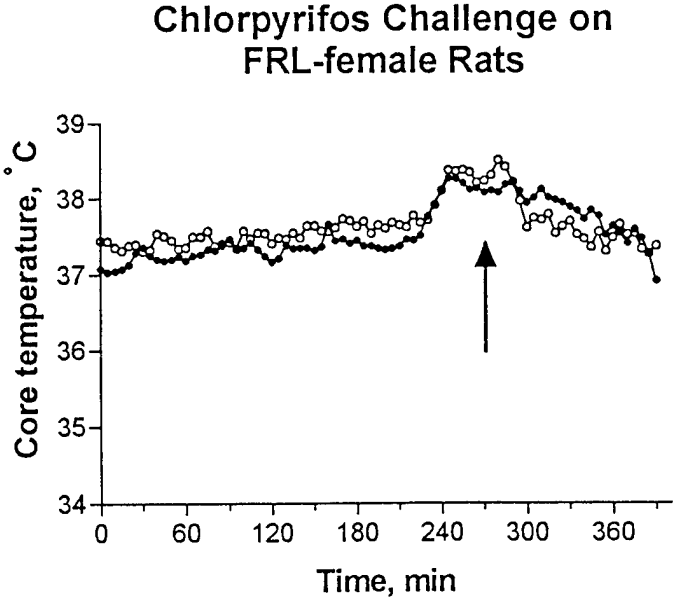
240
300
360
Time, min

F


Chlorpyrifos Challenge on
FRL-female Rats
Core temperature, °C
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38
37
36
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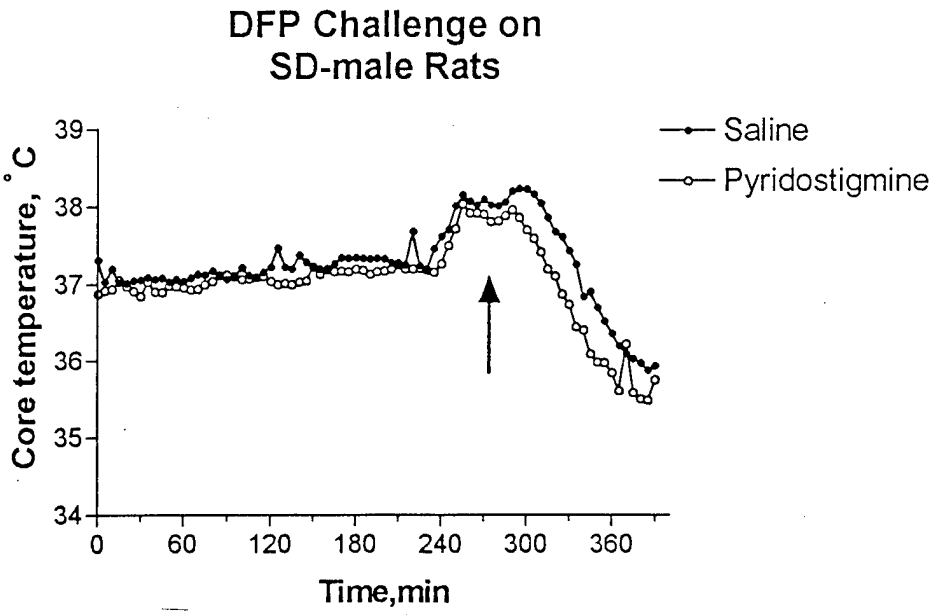
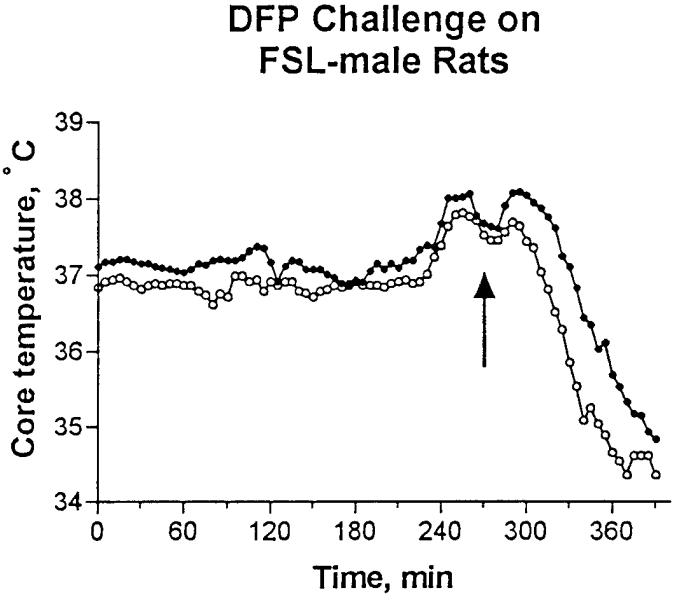
34
0
60
120
180
240
300
360
Time, min

FIGURE 5

A


DFP Challenge on
SD-male Rats
Saline
Pyridostigmine
Core temperature, °C
39
38
37
36
35
34
0
60
120
180
240
300
360
Time,min

B


DFP Challenge on
FSL-male Rats
Core temperature, °C
39
38
37
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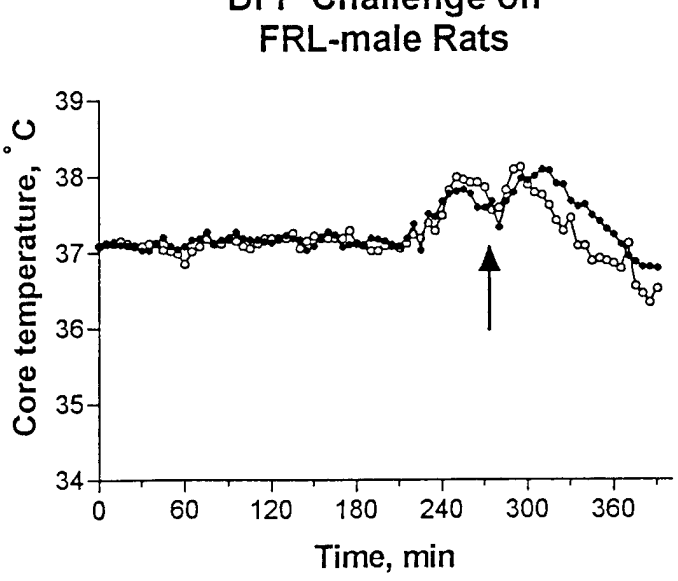
35
34
0
60
120
180
240
300
360
Time, min

C


DFP Challenge on
FRL-male Rats
Core temperature, °C
39
38
37
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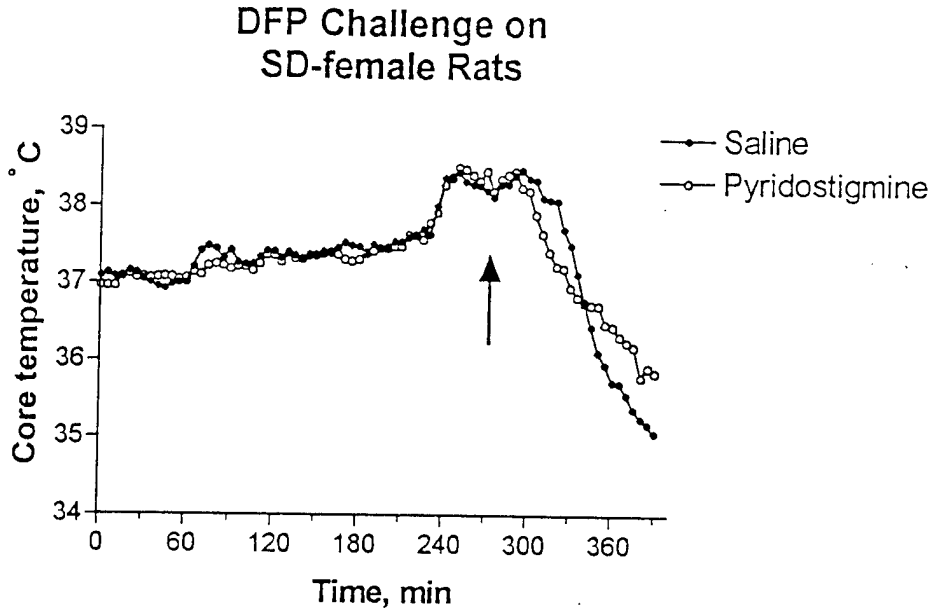
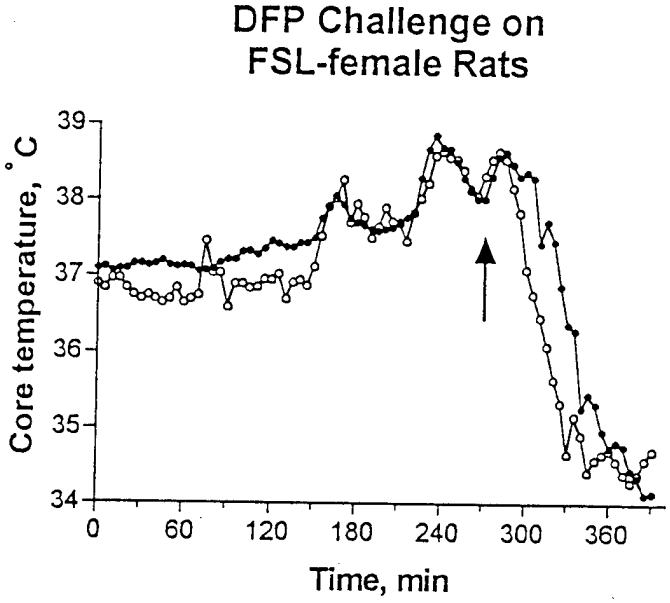
35
34
0
60
120
180
240
300
360
Time, min

FIGURE 5

D


DFP Challenge on
SD-female Rats
Saline
Pyridostigmine
Core temperature, °C
39
38
37
36
35
34
0
60
120
180
240
300
360
Time, min

E


DFP Challenge on
FSL-female Rats
Core temperature, °C
39
38
37
36
35
34
0
60
120
180
240
300
360
Time, min

F

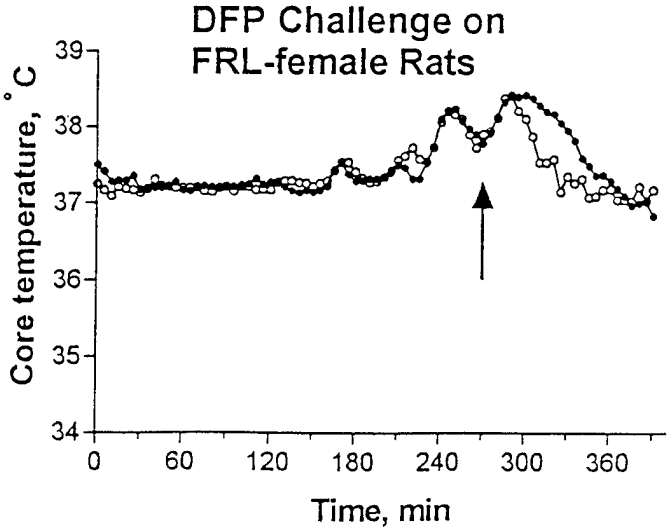
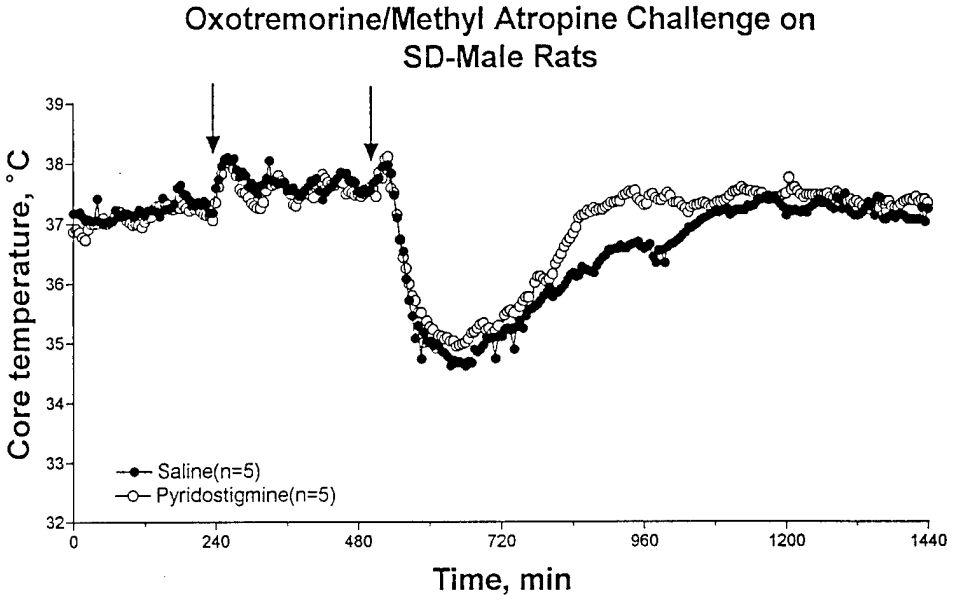
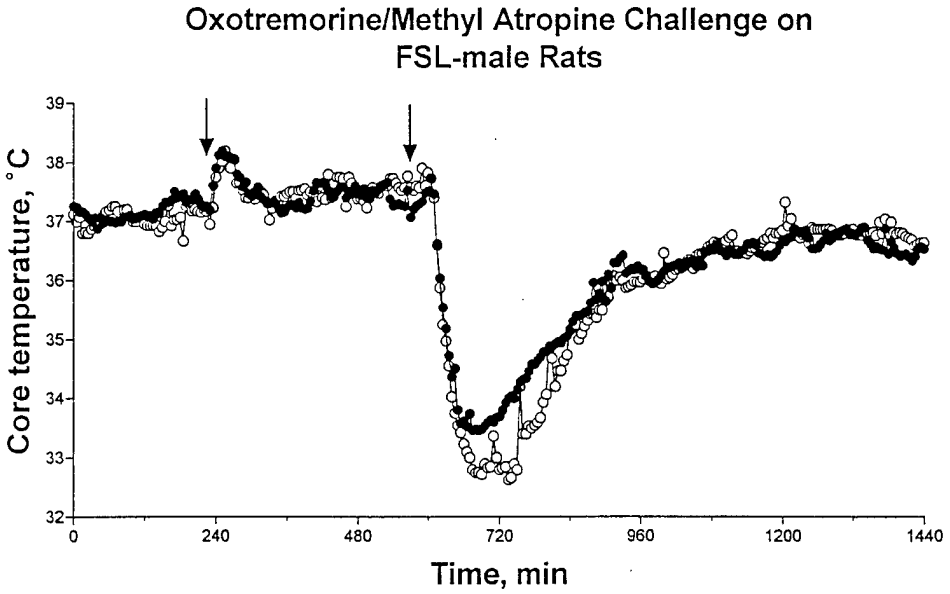

DFP Challenge on
FRL-female Rats
Core temperature, °C
39
38
37
36
35
34
0
60
120
180
240
300
360
Time, min

FIGURE 6

A


Oxotremorine/Methyl Atropine Challenge on
SD-Male Rats
Core temperature, °C
Time, min
Saline(n=5)
Pyridostigmine(n=5)

B


Oxotremorine/Methyl Atropine Challenge on
FSL-male Rats
Core temperature, °C
Time, min

C

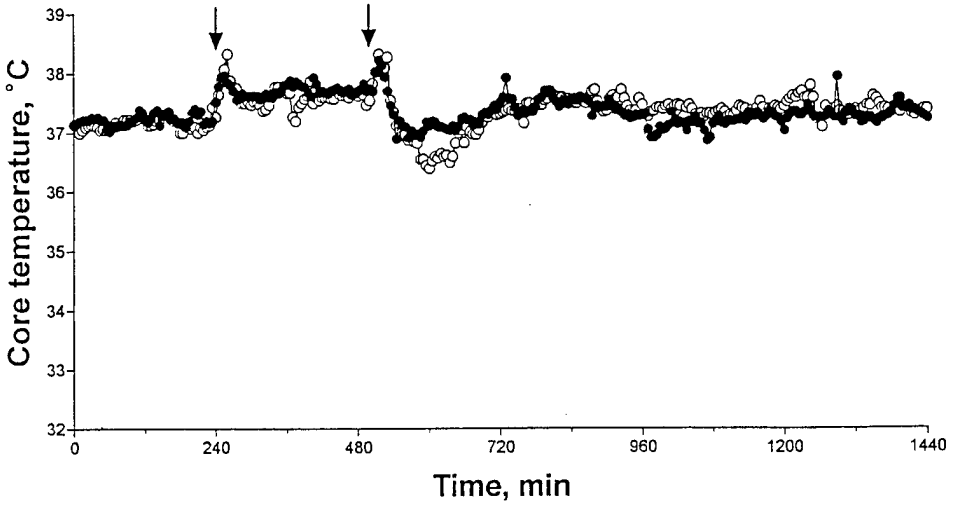
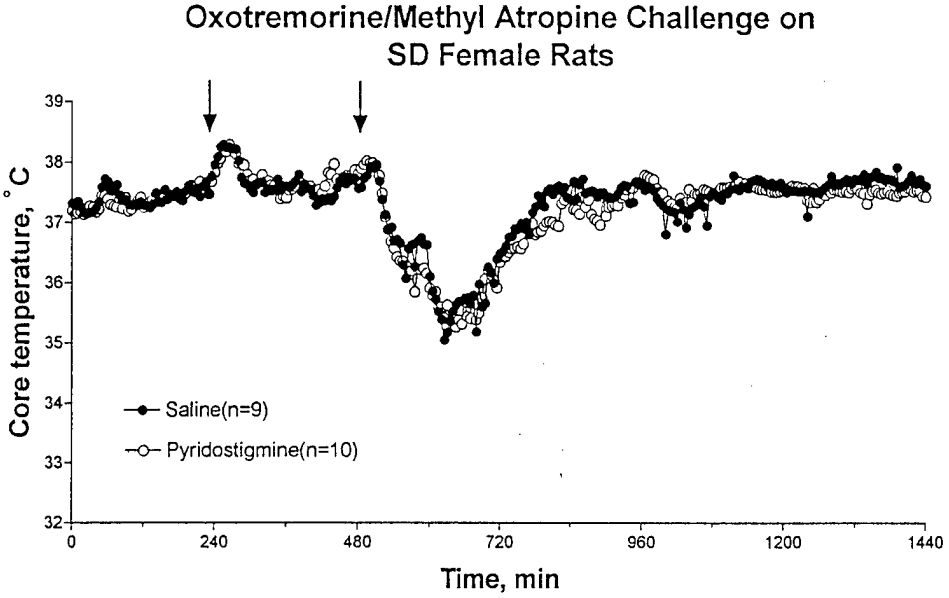
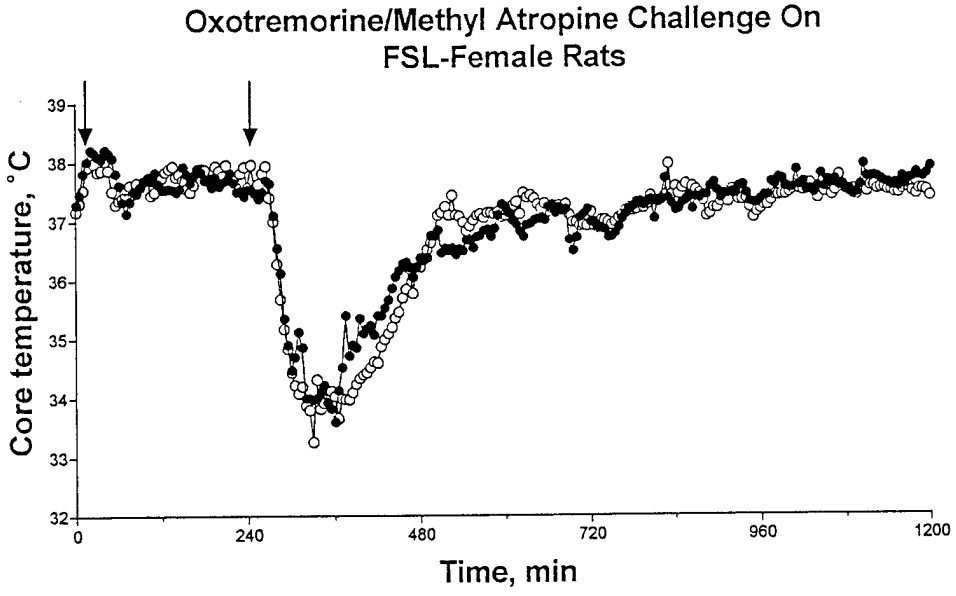

Oxotremorine/Methyl Atropine Challenge on
FRL-Male Rats
Core temperature, °C
Time, min

FIGURE 6

D


Oxotremorine/Methyl Atropine Challenge on
SD Female Rats
Core temperature, °C
Saline(n=9)
Pyridostigmine(n=10)
Time, min

E


Oxotremorine/Methyl Atropine Challenge On
FSL-Female Rats
Core temperature, °C
Time, min

F

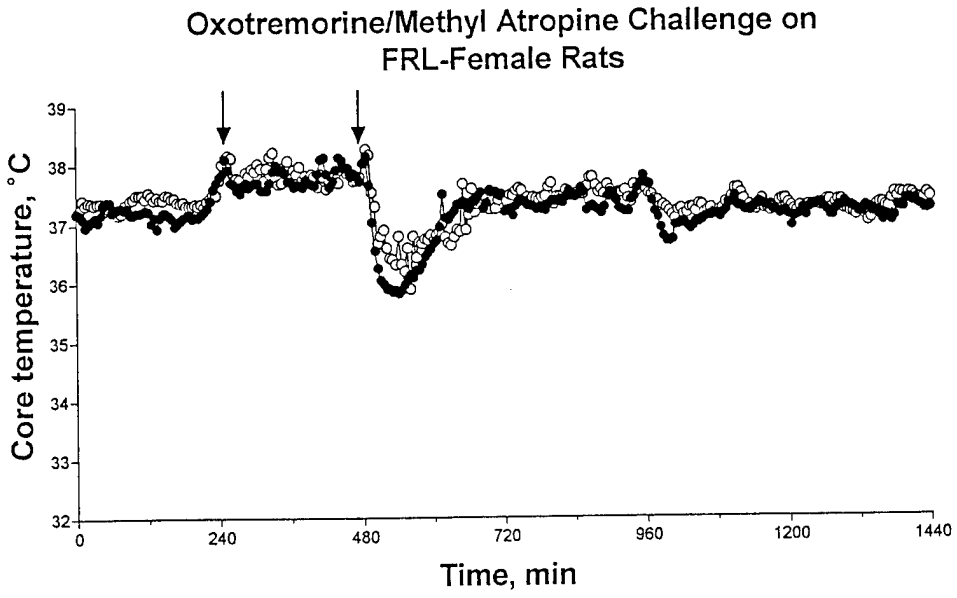
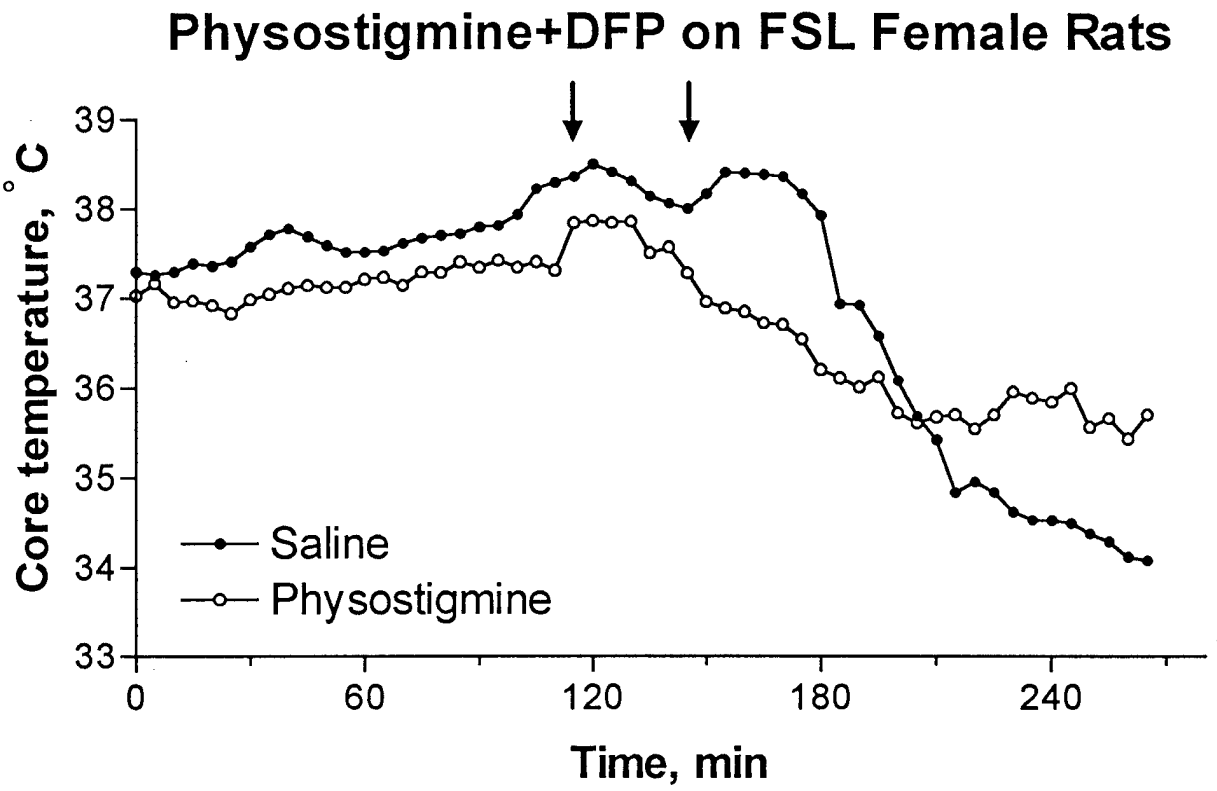

Oxotremorine/Methyl Atropine Challenge on
FRL-Female Rats
Core temperature, °C
Time, min

FIGURE 7

A


Physostigmine+DFP on FSL Female Rats
Core temperature, °C
39
38
37
36
35
34
33
Saline
Physostigmine
0
60
120
180
240
Time, min

B

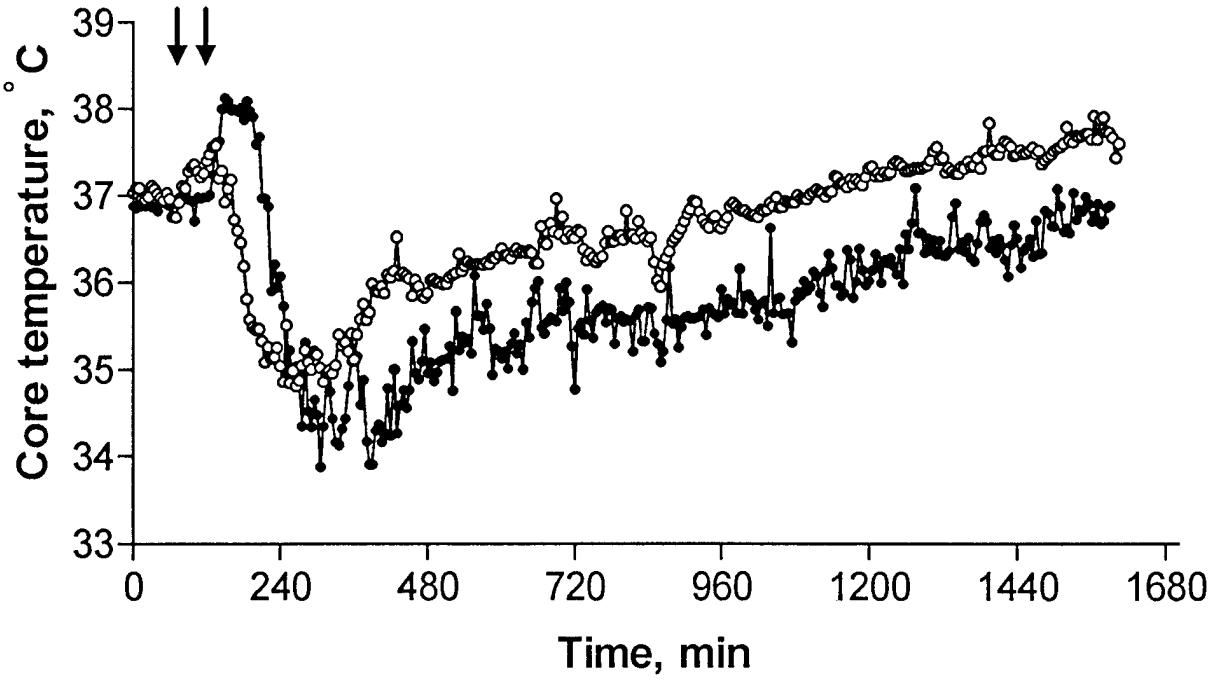
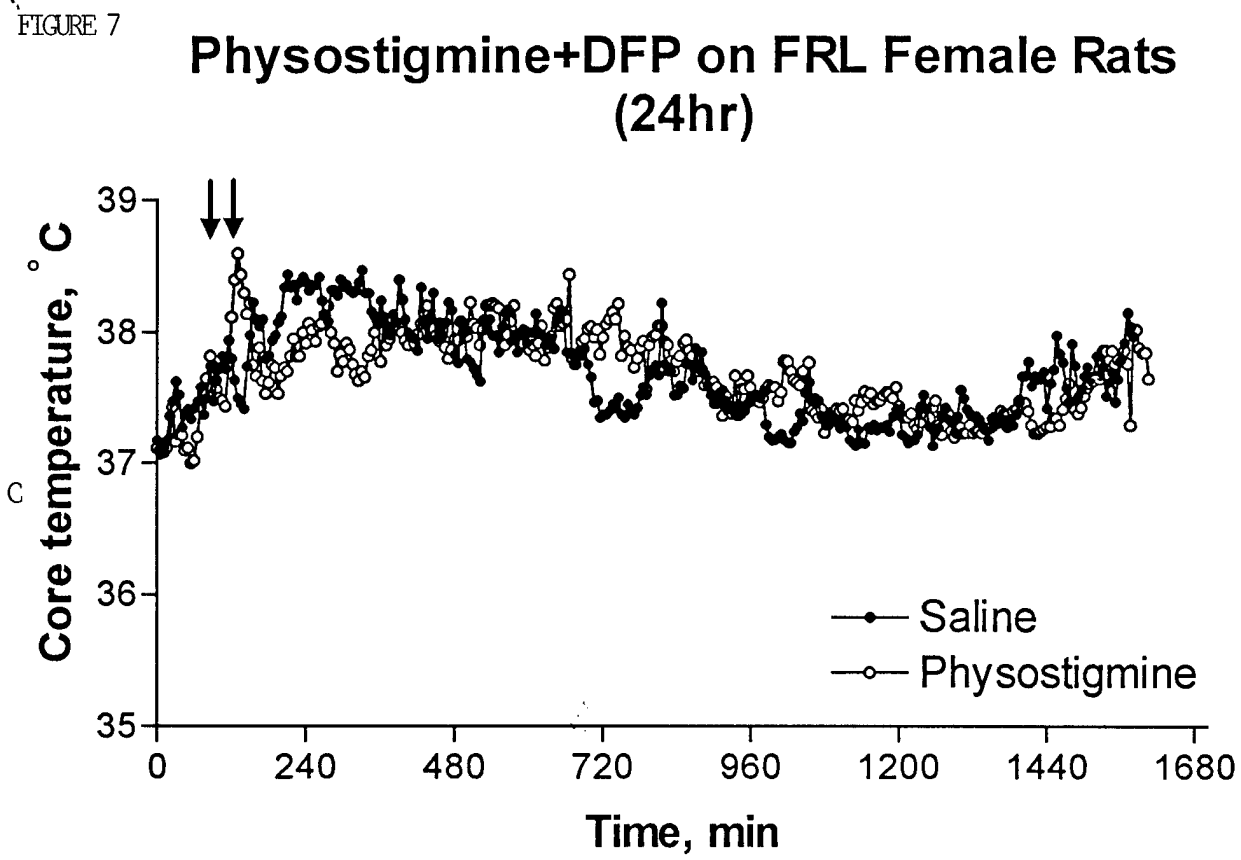
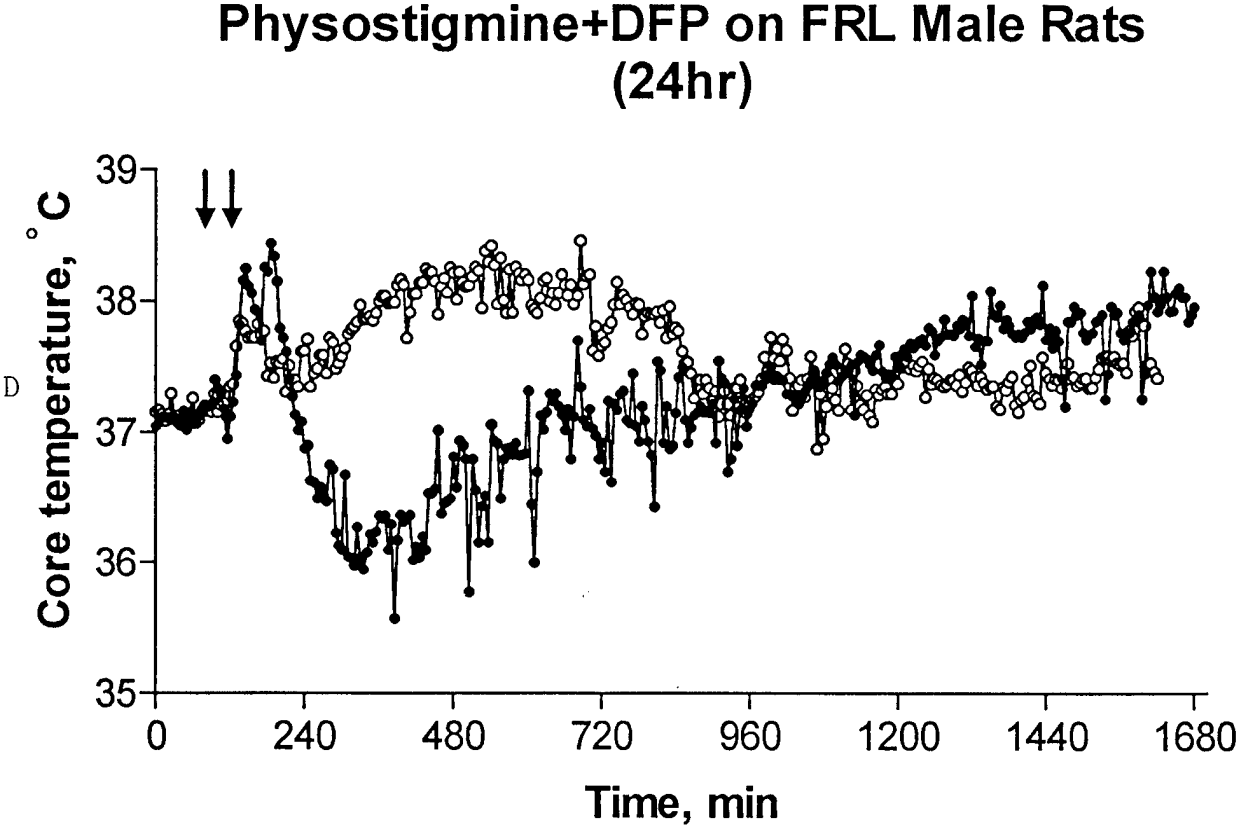

Physostigmine+DFP on FSL Male Rats
(24hr)
Core temperature, °C
39
38
37
36
35
34
33
0
240
480
720
960
1200
1440
1680
Time, min

FIGURE 7


Physostigmine+DFP on FRL Female Rats
(24hr)
Core temperature, °C
39
38
37
36
35
0
240
480
720
960
1200
1440
1680
Time, min
Saline
Physostigmine

C


Physostigmine+DFP on FRL Male Rats
(24hr)
Core temperature, °C
39
38
37
36
35
0
240
480
720
960
1200
1440
1680
Time, min

D

## KEY RESEARCH ACCOMPLISHMENTS

- Pyridostigmine elevates growth hormone differentially in rat strains
- Pyridostigmine does not alter temperature or activity
- Pyridostigmine protects against diarrhea induced by organophosphosphates
- Pyridostigmine does not protect against hypothermia induced by organophosphates
- Pyridostigmine may potentiate the hypothermic effects of centrally acting cholinergic agents.
- Physostigmine may protect against the hypothermic effects of organophosphates.

## REPORTABLE OUTCOMES

The following papers, abstracts and presentations have been supported by this grant.

Research Articles/Book Chapters

Overstreet DH, Rezvani AH, Yang Y, Hamedi H, Janowsky DS. Animal model of chemical sensitivity involving cholinergic agents. In: Toxicology in Risk Assessment. Salem, H. and Olajos, E.J. (eds.) (in press), 1999.

Overstreet DH, Daws LC, Schiller GD, Orbach J, Janowsky DS. Cholinergic/serotonergic interactions in hypothermia: Implications for rat models of depression Pharmacol Biochem Behav 1998;59:777-785..

Overstreet DH, Djuric V. Links between multiple chemical sensitivity and asthma in a rat model of cholinergic hypersensitivity: A brief review. Toxicol Indust Health (in press), 1999, (16 pages).

Overstreet DH. Organophosphate pesticides, cholinergic function and cognitive performance in advanced age. Neurotoxicology (in press), 1999, (22 pages).

Overstreet DH, Yang Y, Rezvani AH, Mason GA, Janowsky DS. Supersensitive growth hormon responses to pyridostigmine in a genetic animal model of depression with cholinergic hypersensitivity. Neuroendocrinology (submitted).

Overstreet DH, Rezvani AH, Clark Ejr, Yang Y. Chronic pretreatment with pyridostigmine potentiates the hypothermic effects of organophosphate anticholinesterases. Neurotoxicology (submitted).

Abstracts

Overstreet DH, Yang Y, Hamedi M, Janowsky DS, Rezvani AH. Strain- and Gender-dependent effects of oxotremorine and pyridostigmine. Presented at 27th Annual Meeting of Society for Neuroscience, New Orleans, LA, October 25-30, 1997, Abstract 728.20.

Overstreet DH, Yang Y, Clark Ejr, Rezvani AH. More rapid decreases in temperature following anticholinesterase challenges in rats pretreatred with pyridostigmine. Presented at 16th International Conference on Neurotoxicology, Little Rock, AR September 14, 1998.

Overstreet DH, Mason GA, Yang Y, Rezvani AH. Supersensitive growth hormone responses following pyridostigmine challenge in a genetic animal model of depression. Presented at 28th Annual Meeting of the Society for Neuroscience, Los Angeles, CA, Nov. 7-12, 1998, Abstract 189.10.

Presentations

Overstreet DH, Rezvani AH, Yang Y, Hamedi H, Janowsky DS. Animal model of chemical sensitivity involving cholinergic agents. Presented at Toxicology in Risk Assessment Symposium held in Bethesda, MD, May 14-16, 1997.

Overstreet DH, Daws LC, Schiller GD, Orbach J, Janowsky DS. Cholinergic/serotonergic interactions in hypothermia: Implications for rat models of depression.

Presented at 4th Annual Symposium by Pharmacology, Biochemistry and Behavior, Morzine, France, January 4-11, 1998.

Overstreet DH, Yang Y, Rezvani AH. Failure of Pyridostigmine Pretreatment to Protect against the Effects of Chlorpyrifos. Presented at Conference on Federally Sponsored Gulf War Veterans' Illnesses Research, Pentagon City, VA, June 17-19, 1998.

Overstreet DH. A Rat Model of Cholinergic Hypersensitivity: Link between Asthma and Multiple Chemical Sensitivity. Presented at the special symposium on Multiple Chemical Sensitivity held at the 216th National Meeting of the American Chemical Society, Boston, MA, August 22-27, 1998.

Overstreet DH. Organophosphate pesticides, cholinergic function and cognitive performance in advanced age. Presented at the 16th International meeting of Neurotoxicology Society, Little Rock, AR, September 13-15, 1999.

Employment/Research Opportunities

Two individuals who received support from this project will be pursuing further educational opportunities. Mr. Elijah Clark, Jr. will be pursuing a Master's Degree in Psychology at Howard University. Mr. Lee Gause will be attending Medical School at East Carolina University. Another individual (Mr. Mani Hamedi) who worked on early aspects of the project but received no financial support is attending medical school at Temple University.

Individuals Supported by the Grant

As required for annual reports, we are listing the personnel who were supported by this grant. We have elected to use the Reportable Outcome Section to report this item.

David H. Overstreet, Ph.D. Amir H. Rezvani, Ph.D. Ying Yang, M.D.

Elijah Clark Jr., B.A. Lee Gause, B.S.

## CONCLUSIONS

The findings reported here are in dramatic contrast to the predominantly negative effects of pyridostigmine described in our first annual report (See summary in Table 2 and figures in Appendix). The elevation of growth hormone induced by pyridostigmine was expected because of the extensive previous literature in both animals and humans. The inverted U shape function, where the levels are highest at the intermediate dose, was not anticipated, but is consistent with what has been reported for many other biological phenomena. At this time we do not have any explanation for why the level of growth hormone is not stimulated by the 36 mg/kg dose of pyridostigmine. It might be related to the general concept that if too great an inhibition of cholinesterase occurs, there is a paralysis of cholinergic transmission rather than a facilitation (Taylor, 1996). Cholinesterase assays were to be performed on serums from all of the treated groups during the final year of this project but these assays could not completed due to a number of circumstances (See later). These values may be helpful in interpreting the dose-dependent effects of pyridostigmine.

The fact that growth hormone was elevated following acute pyridostigmine treatment while core body temperature and activity were not significantly altered suggests that the site of the cholinergic receptors mediating the growth hormone response reside outside of the blood brain barrier. Various investigators have taken advantage of the lack of central effects of pyridostigmine to conduct challenge tests in humans (e.g. Chaudhuri et al., 1997; Ghigo et al., 1993; O'Keane et al., 1992, 1994). Unlike the centrally acting physostigmine, which induces nausea and other side effects, pyridostigmine appears to be well tolerated. We noted a very low incidence of diarrhea when the rats were sacrificed 30 min after oral pyridostigmine treatment in

our first report and the current data, collected in the saline challenge rats 2.5 hour after the chronic pyridostigmine treatment, supports this.

Pretreatment with pyridostigmine chronically for 14 days significantly altered the rate of change in body temperature in the rats challenged with CPF or DFP. However, the direction of the change suggested that the rats were more sensitive to the effects of the challenge agents, not less as would have been predicted by those favoring the use of pyridostigmine as a prophylactic in Gulf War paricipants. The accelerated drop in temperature in the pyridostigmine-treated rats may be related to the blockade or saturation of peripheral cholinesterases by pyridostigmine. Because there are now fewer binding sites for DFP and CPF-oxon in the periphery, these cholinesterase inhibitors more readily enter the central nervous system, where they will induce a decrease in body temperature. Further experiments on the time course of the inhibition of the brain cholinesterase activity in the treated rats may provide a clue to the validity of this argument. Data on brain cholinesterase activity in the animals used in these experiments would not be particularly informative because the temperatures of the treated and untreated groups were similar two hours after treatment (Table 4, Figures 4 and 5).

Another hypothesis to account for the apparently greater sensitivity of the rats pretreated with pyridostigmine is that brain muscarinic receptors may have increased during the chronic treatment with pyridostigmine, as has been reported for the related compound neostigmine (Costa et al., 1982). Brains from each of the treatments have been stored and were to be processed during 1999 to determine whether there have been changes in any of the groups as a consequence of the chronic treatment regimen. However, due to unforeseen circumstances, as described later, these assays have not yet been completed. These studies will also provide a comparison of the FSL and FRL rats with the SD rats. According to the data on temperature,

both the FRL and FSL rats would be predicted to have muscarinic receptor differences from the SD rats The fact that the FSL rats exhibited an increase in responsiveness to OXO, whereas the SD and FRL rats did not, suggests that there might be receptor changes only in this line.

The incidence of diarrhea was very low in all of the groups. This may have been a consequence of the mode of treatment, as the incidence was higher in the rats treated i.m. with DFP than in the rats treated orally with pyridostigmine or CPF. Pyridostigmine did protect against the diarrhea induced by DFP, indicating that the drug does have some prophylactic effects. Since the sites that mediate the growth hormone responses to pyridostigmine also appear to reside outside of the blood brain barrier, one might expect that the growth hormone response to CPF and DFP will also be blunted by pretreatment with pyridostigmine. Blood samples had been collected from the animals undergoing the various treatments and were to be analyzed for growth hormone in 1999. However, repeated requests to obtain hormone kits from NIDDK were not granted.

Several activities scheduled for the final year of this project were not completed due to unexpected personnel changes and a variety of other factors. As indicated above, we were not able to secure hormone kits from NIDDK. In addition, our hormone expert, Dr. George Mason retired in December. Dr. Amir Rezvani, a long-term collaborator on this and other projects, moved to Duke University. Dr. Ying Yang, a postdoctoral research associate, had to return to China in February as her visa expired. We were able to get her visa extended by six months and took advantage of her expertise with the telemetry protocol to conduct an experiment on the effectiveness of physostigmine, a centrally acting carbamate, in protecting against the hypothermic effects induced by DFP. The PI attended a number of conferences last year and presented papers on several topics related to this project; these manuscripts are included in the

Appendix. The cholinesterase receptor binding assays were not completed because we were not able to negotiate the use of laboratory equipment under the control of other investigators in the Center due to the heavy use of the equipment by their personnel. Negotiations are continuing.

In conclusion, on the basis of the present findings, pyridostigmine by itself is not a very effective prophylactic against hypothermia induced by centrally acting anticholinesterase agents. The decrease in temperature after CFP and DFP is more rapid and there are no differences in the peak changes at 2 hr after the injection. The 2-hr time point was selected to maximize our chances of seeing significant changes in all of the key variables we proposed to measure (temperature, growth hormone, cholinesterase activity). We have considered the possibility that 2 hr may have been too short to reveal the protective effects of pyridostigmine and will, therefore, be examining rats for up to 24 hr after their challenge treatments, when temperature is expected to recover. Our current conclusion that pyridostigmine is a relatively ineffective prophylactic against the anticholinesterase agents CPF and DFP is based on our present findings, but they are consistent with the conclusions made by other recent investigators. For example, it has been recently demonstrated that centrally acting physostigmine, administered by mnipump, was more effective than pyridostigmine in protecting against soman (Phillippens et al., 1998). Other investigators have earlier remarked on the greater effectiveness of physostigmine compared to pyridostigmine in protecting against organophosphate poisoning (e.g., Deshpande et al., 1986; Solana et al., 1990). Our limited study with physostigmine also indicated that it had some protective effects against the hypothermia induced by DFP (Fig. 7). However, a consequence of this pretreatment is the likelihood of effects in animals (humans) that might be hypersensitive to cholinergic agents.

Because of the generally more rapid effects of CPF and DFP in PYR-pretreated rats and the supersensitive responses to OXO in the FSL rats pretreated with PYR, there is a possibility that some individuals who had been receiving pyridostigmine as a prophylactic during the Persian Gulf War might have been sensitized to the effects of organophosphates. The possibility that genetic background might interact with prophylactic treatments and accidental exposures to contribute to Gull War Syndrome needs to be further evaluated.

Two experiments in individuals with Gulf War Syndrome can be proposed on the basis of the present findings and other relevant literature. Firstly, these individuals and matched control subjects could be challenged with pyridostigmine and growth hormone levels assessed. Based on the hypothesis that the individuals with Gulf War Syndrome have a cholinergic hypersensitivity similar to that seen in the FSL rats, one would predict that they would exhibit larger increases in growth hormone than their control counterparts. Since the control subjects would be matched as closely as possible for exposure to OPs, a difference between the groups could be related a genetic predisposition.

A second experiment is derived from the literature on growth hormone indicating exaggerated responses in several abnormal conditions in humans (e.g., Cooney et al., 1997b). Because these disorders also have a genetic component, it would be predicted that relatives of individuals with Gulf War Illness would have a greater incidence of depressive disorders, for example, than relatives of control subjects. To our knowledge such an analysis of relatives has not yet been performed. Recent studies of individuals with Gulf War Illness have reported a variety of complaints, which may include psychiatric symptoms (Sillanpaa et al., 1999; Haley et al., 1997).

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## APPENDICES

The appendices consist of copies of the following papers and abstracts.

Overstreet DH, Rezvani AH, Yang Y, Hamedi H, Janowsky DS. Animal model of chemical sensitivity involving cholinergic agents. In: Toxicology in Risk Assessment. Salem, H. and Olajos, E.J. (eds.) (in press), 1999.

Overstreet DH, Daws LC, Schiller GD, Orbach J, Janowsky DS. Cholinergic/serotonergic interactions in hypothermia: Implications for rat models of depression Pharmacol Biochem Behav 1998;59:777-785..

Overstreet DH, Djuric V. Links between multiple chemical sensitivity and asthma in a rat model of cholinergic hypersensitivity: A brief review. Toxicol Indust Health (in press), 1999, (16 pages).

Overstreet DH. Organophosphate pesticides, cholinergic function and cognitive performance in advanced age. Neurotoxicology (in press), 1999, (22 pages).

Overstreet DH, Yang Y, Rezvani AH, Mason GA, Janowsky DS. Supersensitive growth hormon responses to pyridostigmine in a genetic animal model of depression with cholinergic hypersensitivity. Neuroendocrinology (submitted).

Overstreet DH, Rezvani AH, Clark Ejr, Yang Y. Chronic pretreatment with pyridostigmine potentiates the hypothermic effects of organophosphate anticholinesterases. Neurotoxicology (submitted).

Overstreet DH, Yang Y, Hamedi M, Janowsky DS, Rezvani AH. Strain- and Gender-dependent effects of oxotremorine and pyridostigmine. Presented at 27th Annual Meeting of Society for Neuroscience, New Orleans, LA, October 25-30, 1997, Abstract 728.20.

Overstreet DH, Yang Y, Clark Ejr, Rezvani AH. More rapid decreases in temperature following anticholinesterase challenges in rats pretreatred with pyridostigmine. Presented at 16$^{th}$ International Conference on Neurotoxicology, Little Rock, AR September 14, 1998.

Overstreet DH, Mason GA, Yang Y, Rezvani AH. Supersensitive growth hormone responses following pyridostigmine challenge in a genetic animal model of depression. Presented at 28$^{th}$ Annual Meeting of the Society for Neuroscience, Los Angeles, CA, Nov. 7-12, 1998, Abstract 189.10.

Overstreet DH, Yang Y, Rezvani AH. Failure of Pyridostigmine Pretreatment to Protect against the Effects of Chlorpyrifos. Presented at Conference on Federally Sponsored Gulf War Veterans' Illnesses Research, Pentagon City, VA, June 17-19, 1998.

# ANIMAL MODEL OF CHEMICAL SENSITIVITY INVOLVING CHOLINERGIC AGENTS

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Abstract

Risk assessment procedures need to take into account the possibility of individual differences in drug sensitivity. To illustrate this point this paper will summarize data collected on the Flinders Line rats, which are differentially sensitive to a variety of chemical agents, including cholinergic agonists. The Flinders Line rats were developed at Flinders University in Australia by selective breeding for differential responses to the anticholinesterase, diisopropyl fluorophosphate (DFP). Separation of two lines, the Flinders Sensitive Line (FSL) and the Flinders Resistant Line (FRL), was apparent by 8 generations, with the FSL rats being more sensitive to the hypothermic effects of DFP. Subsequently, it was determined that the FSL rats were also more sensitive to directly acting muscarinic agonists, such as oxotremorine and pilocarpine. This increased sensitivity to DFP and muscarinic agonists might be related to the muscarinic receptor elevations seen in the hippocampus, striatum, and hypothalamus of the FSL rats. Because increased sensitivity to muscarinic agonists in the FSL rats is comparable to that seen in depressed humans, various behavioral tests were conducted and the data from these were consistent with the hypothesis that the FSL rats may be a genetic animal model of depression: they are less active in a novel open field, have lower appetites and body weights, are more sensitive to stressors, and their behavioral immobility is ameliorated by chronic treatment with antidepressants. The FSL rats have also been determined to be more sensitive to the effects of a variety of other drugs, including alcohol, diazepam, nicotine, and 8-OH-DPAT, a 5-HT1A receptor agonist. This increased sensitivity to a variety of drugs in FSL rats is reminiscent of human patients suffering from multiple chemical sensitivity (MCS) and suggests that MCS might arise, in part, from genetically influenced muscarinic supersensitivity. The heightened sensitivity of the FSL rats to a variety of drugs suggests that they will also be more sensitive to the effects of pyridostigmine, an anticholinesterase which was given to gulf war participants. The results of initial experiments indicate that there are no differences in hypothermia after pyridostigmine, but FSL rats may be more sensitive to the bradycardia induced by pyridostigmine.

Key Words: Animal Model of MCS; Organophosphate DFP; FSL Rats; Human Depressives;

Cholinergic Supersensitivity; Gulf War Illness

## Introduction

In the assessment of risk to individuals exposed to known or potential toxicological agents. there needs to be a consideration of the possibility that especially sensitive populations exist. For example, some individuals have reported side effects after taking pyridostigmine to protect them against potential nerve gas exposure and others have not. Other individuals have reported increased sensitivity to a variety of chemical agents, usually after a triggering exposure to a specific chemical such as an organophosphate pesticide (e.g., Miller and Mitzel, 1995). The hypothesis that a genetically based cholinergic supersensitivity might underlie the increased sensitivity of these vulnerable human populations will be addressed in the present communication by describing in detail the features of an animal model with cholinergic supersensitivity which is also more sensitive to a variety of drugs and other chemical agents and which may, therefore, mimic the human condition labelled Multiple Chemical Sensitivity (MCS). In the final section of this paper some initial results on the effects of pyridostigmine on this animal model will be presented.

The validity of an animal model rests in part on its similarity in structure and function to a target condition in humans. The closer the similarity to the human condition the model is, the greater is the probability that manipulations of one will provide information valid for extrapolation to the other. A final test of validity comes when predictions made from the animal model and applied to the human condition are shown to be accurate. To evaluate the model proposed below, it is important to summarize the observed clinical characteristics of MCS.

## MULTIPLE CHEMICAL SENSITIVITY

Multiple Chemical Sensitivity (MCS) is a syndrome in which, following acute or repeated exposure to one or more chemicals, most commonly organophosphate pesticides (OPs), individuals become overly sensitive to a wide variety of chemically-unrelated compounds. These can include ethanol, caffeine and other psychotropic drugs (Ashford and Miller, 1989, 1991; Bell et al., 1992; Cullen, 1987; Miller, 1994). The symptoms of MCS often reported include fatigue, cognitive

difficulties, depression, irritability, headaches, dyspnea, digestive problems, musculoskeletal pain, and numbness in their extremities. These conditions often overlap those of common medical illnesses such as depression, somatization disorder, chronic fatigue syndrome, fibromyalgia, asthma and others. However, a distinguishing feature of MCS is the strong belief of the patients that their symptoms are brought on by common exposures to low levels of volatile organic chemicals such as fragrances, insecticides, traffic exhaust, disinfectants and perfumes.

Descriptions of MCS have been noted in various journals for more than 40 years. In recent years, occupational medicine physicians in universities have reported seeing increasing numbers of individuals who appear to have it. In addition, there have been three federally-sponsored workshops focussed on MCS (Association of Occupational and Environmental Clinics, 1992; National Research Council, 1992; Mitchell and Price, 1994). Sponsoring agencies have included The National Research Council (NRC), the Agency for Toxic Substances and Disease Registry (ATSDR), the Environmental Protection Agency, and the National Institute of Environmental Health Sciences (NIEHS). The recommendations from these meetings have repeatedly stressed the need for further research on the condition and the development of animal models.

MCS has been described as a two-step process that is analogs to but different from the process that occurs in allergic diseases (Ashford and Miller, 1991): For both allergies and MCS there is *Induction* (initiation, sensitization or loss of tolerance) as a consequence of an initial chemical exposure or to sensitization to bee venom, for example. In both conditions, there is also subsequent *triggering* of symptoms; however, in MCS this may occur from exposure to a wide range of chemically-diverse substances, while in allergy antibodies are highly specific and spreading of sensitivities to chemically unrelated substances does not occur.

MCS patients most frequently report their condition as being induced by pesticides, especially OPs and carbamates (Ashford and Miller, 1991; Miller and Mitzel, 1995). Significantly, exposures to OP and carbamate agents during the Gulf War included pesticides, pyridostigmine bromide (used as a prophylaxis for nerve agents), and, possibly, low levels of actual nerve agents. Although chemicals in this class can inhibit cholinesterase, rarely have cholinesterase levels been

measured in sporadic MCS cases, and frequently symptoms typically associated with cholinesterase inhibition are absent among individuals who report ultimately developing MCS as a consequence of OP exposure. While acute OP toxicity has generally been considered to be reversible, provided it is not fatal, the toxicology literature contains a variety of examples of individuals who were exposed to these agents and later showed persistent psychological, psychiatric, or neuropsychological deficits (Gershon and Shaw, 1961; Rosenstock et al., 1991; Rowntree at al., 1950; Savage et al., 1988; Tabershaw and Cooper, 1966). To account for these long-lasting effects it has been proposed that OPs may damage cholinergic receptors or in other ways induce injury independent of their ability to inhibit cholinesterase (Gupta and Abou-Donia, 1994; Huff et al., 1994).

Several case reports of individuals developing MCS after exposure to pesticides (Rosenthal and Cameron, 1991; Cone and Sult, 1992) have appeared recently. Even more recently, Miller and Mitzel (1995) surveyed 112 MCS patients, 37 of whom attributed their illness to exposure to an OP or carbamate pesticide and the other 75 to remodelling of a building a procedure which commonly involves exposures to low levels of mixed solvents emanating from fresh paint, carpeting, glues, etc. Following their initial exposure, both groups reported similar symptoms and similar intolerances to chemicals, foods, ethanol, and caffeine. However, overall, the pesticide-exposed group reported significantly greater symptom severity. The authors interpreted these findings as suggesting a possible common pathway for the development of MCS, despite the fact that the two groups initially experienced exposures to very different classes of chemicals. They hypothesized that the relatively greater neurotoxicity and/or potency of the cholinesterase inhibitors as compared to mixed low-level solvents might account for the greater symptom severity in the pesticide-exposed individuals.

An important observation in this field is that MCS patients usually report that other individuals simultaneously exposed to similar amounts of pesticides, e.g, family members, friends, or co-workers, did not develop MCS or even experience transient illness. This observation suggests that a subset or subsets of the people may be more vulnerable to developing MCS. Indeed, some (Black et al., 1990; Simon et al., 1990), but not all (Fiedler et al., 1992) researchers have reported

greater rates of depression and somatization disorder predating the "initiating" chemical exposure among persons with MCS as compared to controls. Thus, any model must take into account why only some individuals develop MCS after exposures to pesticides or other chemicals.

One such model which will be described in the subsequent sections of this paper is the FSL (Flinders Sensitive Line) rat. This rat was developed by selective breeding for increased sensitivity to an OP, so it shares some etiological similarity to patients with MCS who were exposed to pesticides.

## AN ANIMAL MODEL

The FSL rat model is one with which we have had extensive experience, particularly in research on depressive syndromes (Overstreet, 1993; Overstreet and Janowsky, 1991; Overstreet et al., 1995). Analogies between depressed states and MCS, as well as substance hypersensitivities in FSL rats, first brought our attention to the potential value of this model for experimental studies of MCS, as recently described (Overstreet et al., 1996). Further, because the FSL rats were selectively bred for increased responses to the organophosphate, DFP, it is possible that they may have some special relevance to Gulf War Illness, commonly reported in individuals exposed to the carbamate, pyridostigmine. Some preliminary findings of our work with pyridostigmine will be presented in the final section of this paper.

### Selective Breeding for OP Differences

The FSL rat model arose from a selective breeding program designed to produce two lines of rats, one with high (FSL) and one with low (Flinders Resistant Line - FRL) sensitivity to the anticholinesterase agent, diisopropylfluorophosphate (DFP) (Overstreet et al., 1979; Russell et al., 1982). The selective breeding program, which was initiated at Flinders University in Adelaide, Australia, utilized three somatic measures of DFP (Overstreet et al., 1979; Russell et al., 1982). A rank-order system was used to give equal weighting to each of the three variables. Rats which had the lowest average ranks were intermated to establish and maintain the line of more sensitive rats (FSL), while rats which had the highest average ranks were intermated to establish and maintain the

line of more resistant rats (FRL). Subsequent studies showed that randomly bred Sprague-Dawley rats, from which the lines were originally derived, were not different from the FRL rats. On the other hand, FSL rats were significantly more sensitive to DFP than the other two groups (Overstreet et al., 1979; Russell et al., 1982).

Biochemical Mechanisms

This project was initiated, in part, to develop genetically resistant lines of rats so that the biochemical mechanisms of resistance could be compared with those of tolerance. Early studies ruled out changes in acetylcholinesterase as a mechanism to account for the differential sensitivity of FSL and FRL rats to DFP (Overstreet et al., 1979; Russell and Overstreet, 1987; Sihotang and Overstreet, 1983), just as has been found for tolerance development (See Russell and Overstreet, 1987). Because DFP-tolerant rats were subsensitive to the effects of muscarinic agonists (e.g., Overstreet et al., 1973, 1974), the effects of muscarinic agonists on the FSL and FRL rats were examined (Overstreet 1986; Overstreet and Russell, 1982; Overstreet et al., 1986a,b). These studies showed that the FSL rats were more sensitive to pilocarpine, arecoline and oxotremorine than were the FRL rats; this supersensitivity was seen for a variety of responses, including hypothermia, reduced locomotor activity, and suppression of bar-pressing for water reward (Overstreet and Russell, 1982). Thus, FSL rats, developed by selectively breeding for increased sensitivity to DFP, exhibited opposite changes in sensitivity to muscarinic agonists compared to DFP-tolerant rats.

Biochemical studies indicated that the FSL rats exhibited greater numbers of muscarinic receptor binding sites in the hippocampus and striatum than the FRL rats (Overstreet et al., 1984; Pepe et al., 1988)., but there were no differences in acetylcholine turnover (Overstreet et al., 1984). Thus, once again, the FSL rats appear to represent the converse of DFP-tolerant rats; having increased numbers of receptors rather than reduced numbers (See Russell and Overstreet, 1987). It appears that both tolerance and acute sensitivity to cholinergic agents is related to postsynaptic cholinergic mechanisms rather than presynaptic. Although in both instances, there have been detectable changes in the muscarinic receptors themselves, there are some findings, such as the

increased sensitivity of FSL rats to noncholinergic agents (See Section below), which suggest that post-receptor mechanisms may also contribute.

Behavioral Features of FSL Rats

The FSL and FRL rats differ on a large number of behavioral tasks, as recently summarized in several review papers (Overstreet et al., 1995, 1996). In this section we will highlight a number of the key differences. The FSL rats have been reported to have lower locomotor activity than the FRL rats under a number of experimental conditions (Bushnell et al., 1995; Overstreet, 1986; Overstreet and Russell, 1982) but not all (Criswell et al., 1994; Rezvani et al., 1994). They are even less active when stressed prior to exposure to the open field (Overstreet, 1986; Overstreet et al., 1989a).

Results from several other behavioral paradigms are consistent with the view that depressive-like psychomotor retardation symptoms are more apparent in the FSL rats after exposure to stressors. For example, the FSL rats are impaired in active avoidance paradigms compared to the FRL rats (Overstreet and Measday, 1985; Overstreet et al., 1990a, 1992a). Another stress-oriented paradigm which has provided important information about behavioral differences between FSL and FRL rats is the forced swim test. Upon initial exposure in a cylinder (18-20 cm diameter) of water (25 $^{o}$C), FSL rats are more immobile than the FRL rats (Overstreet, 1986; Overstreet et al., 1986a, Pucilowski and Overstreet, 1993; Schiller et al., 1992). This exaggerated immobility of the FSL rats is counteracted by chronic but not acute treatment with antidepressants (Overstreet, 1993; Pucilowski and Overstreet, 1993; Schiller et al., 1992). These findings provide further support for the contention that the FSL rat is a useful animal model of depression.

There are also differences in reward-related behaviors between the FSL and FRL rats which are consistent with the proposal that the FSL rats are a model of depression. In operant bar-pressing tasks, the FSL rats bar-pressed at lower rates and had to be maintained at a lower percentage of their free-feeding body weight and have smaller food pellets (37 vs. 45 mg) in order to keep their motivation sufficiently high to complete the session (Bushnell et al., 1995; Overstreet and Russell,

1982)). Despite these differences in reward-related and stress-related behaviors, there appears to be no differences between the FSL and FRL rats in the ability to perform a matching-to-sample task (Bushnell et al., 1995). However, this test was carried out under normal, unstressed conditions, and it is not clear whether similar findings would obtain under stressed conditions. For example, FSL and FRL rats have similar amounts of saccharin consumption under baseline conditions, but the FSL rats exhibit greater decreases after exposure to chronic mild stress (Pucilowski et al., 1993).

The FSL rats also have elevated REM sleep and reduced latency to REM sleep (Shiromani et al., 1988, Benca et al., 1996), as has been reported in human depressives (Benca et al., 1992) Human depressives are also more sensitive to the effects of cholinergic agonists on REM sleep latency (Janowsky et al., 1994), but there are no data in the FSL rats regarding drug effects on sleep..

In sum, the FSL rats and depressed humans exhibit a large number of behavioral and physiological similarities (See Overstreet, 1993; Overstreet et al., 1995, 1996, for more detailed accounts).

Multiple Chemical Sensitivity in FSL Rats

Clinical observations suggest that MCS may be initiated by acute or chronic exposure to a variety of chemical agents (Miller and Mitzel, 1995). Because the FSL rats were selectively bred to have increased responses to the anticholinesterase agent, DFP, it should not be surprising that they exhibited increased sensitivity to muscarinic agonists (Daws et al., 1991; Overstreet, 1986; Overstreet and Russell, 1982; Overstreet et al., 1992a,b; Schiller et al., 1988). It has also been reported that human depressives are also more sensitive to directly acting muscarinic agonists (Gann et al., 1992; Gillin et al., 1991) as well as anticholinesterases (Gann et al., 1992; Janowsky and Risch, 1987; Nurnberger et al., 1989; O'Keane et al., 1992; Schreiber et al., 1992; Sitaram et al., 1987). A similar increased sensitivity to anticholinesterases has been observed in MCS patients (Cone and Sult, 1992; Miller and Mitzel, 1995; Rosenthal and Cameron, 1991).) but there are no published data for MCS patients regarding sensitivity to direct cholinergic agonists. FSL rats are

also more sensitive to nicotine, which interacts with nicotinic cholinergic receptors (Schiller and Overstreet, 1993).

The cholinergic system interacts with many other major neurotransmitter systems, including serotonergic, dopaminergic, GABAergic, and noradrenergic. Having animals with clear-cut differences in the cholinergic system afforded us the opportunity to test how the FSL and FRL rats differ in response to drugs interacting with these other neurotransmitter systems. Evidence from various drug challenge studies, in which relatively selective drugs are given to FSL and FRL rats, have revealed a substantial number of differences between the FSL and FRL rats, as summarized in Table 1. FSL rats were found to exhibit a greater degree of hypothermia after a variety of drugs which interact with the serotonin 5-HT1A receptor (Wallis et al., 1988; Overstreet et al., 1992a, 1994). This outcome is consistent with much of the evidence suggesting supersensitive serotonergic mechanisms in depressives (Arango et al., 1990; Arora and Meltzer, 1989; Mikuni et al., 1991), but is not consistent with neuroendocrine studies reporting blunted responses to serotonergic agonists, which suggests serotonergic hyposensitivity (Lesch et al., 1990; Meltzer and Lowy, 1987). There are no data on the effects of selective serotonergic agents in MCS patients, but there is one report of supersensitive responses in individuals with chronic fatigue syndrome, which is related to MCS (Bakheit, et al., 1995).

To date no evidence has been obtained to indicate any differences in responses to noradrenergic agents in the FSL rats (Overstreet, 1989; Overstreet et al, 1989a). In contrast, there are quite a number of differences with regard to dopaminergic agents (Table 1). The FSL rats are supersensitive to the hypothermic (Crocker and Overstreet, 1991) and aggression-promoting (Pucilowski et al., 1991a) effects of apomorphine, a mixed D1/D2 agonist, and quinpirole, a selective D2 agonist. On the other hand, the FSL rats were subsensitive to the stereotypy-inducing effects of similar doses of the same compounds and there were no apparent differences in dopamine D2 receptors between FSL and FRL rats (Crocker and Overstreet, 1991). These opposite changes in sensitivity in the various functions might be related to the type of modulation of these functions by the cholinergic and dopaminergic systems. Stimulation of both cholinergic and dopaminergic

systems promotes hypothermic and aggressive responses (Cox et al., 1980; Pucilowski, 1987; Ray et al., 1989), but cholinergic stimulation reduces activity and stereotypy, thereby opposing the effects of dopaminergic stimulation (Fibiger et al., 1970; Klemm, 1989).

The FSL and FRL rats are differentially sensitive to the effects of several pharmacological agents which have modulatory roles at the GABA-A receptor, as summarized in Table 1. However, as with the case of dopamine agonists, the differential effects are observed only for some actions of the drugs, not for all. For example, the hypothermic effects of ethanol are significant higher in the FSL rats compared to the FRL rats, but the sedative effects are similar (Overstreet et al., 1990b). Similarly, the behavioral suppressant effects of diazepam are significantly greater in the FSL rats (Pepe et al., 1988), but its anxiolytic effects in the two lines are comparable (Schiller et al., 1991). The fact that these two commonly abused psychotropic drugs both modulate GABA function at the GABA-A receptor suggests that there might be differences in GABA-A receptor subtype composition between the two lines, but there is not biochemical evidence for such differences as yet. Furthermore, despite differences in sensitivity to the hypothermic effects of ethanol, the FSL and FRL rats do not differ in their rates of voluntary ethanol consumption (Overstreet et al., 1992a).

In summary, it appears that the FSL rat is more sensitive to a variety of chemical agents in addition to the OP anticholinesterase agent for which they were selectively bred. In this regard, the FSL rat is somewhat analogous to MCS patients who have become more sensitive to a range of agents following exposure to OP anticholinesterases. The extent of the similarity between the FSL rats and MCS patients, on one hand, and human depressives and MCS patients, on the other, will be further evaluated in the next section.

## FSL RATS RESEMBLE MCS AND DEPRESSED PATIENTS

As Table 2 summarizes, the behavioral features of individuals with MCS and those of depressed patients and FSL rats are strikingly similar in regard to weight, appetite, activity and stressability, hedonia, and sleep. There are also some uncertainties in Table 2, suggesting several studies that might be carried out in MCS patients to test further the extent of the associations among

the three groups. For example, polysomnographic recordings of sleep in asymptomatic MCS patients would be particularly informative, especially since there is evidence that the REM sleep changes seen in depressed patients may be a trait marker of this disorder (Benca et al., 1992; Janowsky et al., 1994). Since REM sleep alterations can also be related to altered cholinergic mechanisms in general (Shiromani et al., 1987; Janowsky et al., 1994), a finding of REM sleep changes in MCS patients would suggest that altered cholinergic mechanisms might underlie abnormal sensitivity to chemicals. Such a finding would also be consistent with a cholinergic hypothesis as one possible explanation for the similarity between the MCS patients and depressives.

Another similarity between MCS and depressed patients is the ratio of females to males affected: There are many more females than males expressing the symptoms (Table 2). In general, twice as many females than males report depressive symptoms (Goodwin and Jamison, 1990). Similarly, the ratio of female to male MCS patients reaches 4/1 in some studies (Miller and Mitzel, 1995). Again, there is some parallel between the rats and humans because adult female FSL rats are more sensitive to cholinergic agonists than their male counterparts (Netherton and Overstreet, 1983). The possible greater sensitivity of adult females to cholinergic agonists might therefore partially account for the greater incidence of depression (Overstreet et al., 1988) and MCS in women.

Given the behavioral similarities between MCS patients and those who are depressed (Table 2), it is likely that depressed patients might be hypersensitive to similar drugs. Unfortunately, as described in Table 3, there is very little information about the sensitivity of depressed individuals to the range of drugs reported to cause problems in MCS patients, other than depressives' supersensitivity to anticholinesterases and cholinergic agonists (Janowsky et al., 1994). There is somewhat more evidence for a general increase in sensitivity to drugs in the FSL rats (Tables 1 & 3). It is particularly noteworthy that the FSL rats are more sensitive to both alcohol (Overstreet et al., 1990b) and nicotine (Schiller and Overstreet, 1993). The information on the effects of alcohol and nicotine in depressed patients is more complex, as implied by the question mark in Table 3. There are many studies reporting an interaction of depression with primary alcoholism on one hand

(e.g., Kendler et al., 1993; Maier et al., 1994; Schuckit, 1986) and an interaction of smoking with depression on the other (Breslau et al., 1991; Glassman, 1993). Indeed, smoking cessation leads to depression in remitted depressives ((Glassman, 1993). However, we are not aware of any studies specifically stating that depressed patients report intolerances for alcohol and/or nicotine.

It should be stressed that FSL rats may also be less sensitive to certain drugs (Crocker and Overstreet, 1991; Pucilowski et al., 1991). Furthermore, depressed patients exhibit blunted hormonal responses to a number of drugs affecting serotonergic and noradrenergic systems (Meltzer and Lowy, 1987). Consequently, more data from depressed individuals and FSL rats must be collected on their sensitivities to a broader range of chemicals. If the cholinergic system supersensitivity is one mechanism underlying MCS, depression and the FSL rats, then it would be predicted that both FSL rats and depressed individuals would be more sensitive to such drugs. What is also needed are additional data on depressed individuals and FSL rats with respect to the triggering of symptoms by chemical or food exposures (Table 3).

Although we have emphasized the strong possibility of a cholinergic link between MCS patients, depressed patients, and FSL rats, other neurotransmitter systems may be involved. Serotonin has been implicated in depression (Meltzer and Lowy, 1987) and recent experiments on the Flinders rats suggest that serotonergic mechanisms may play an important role in some of their altered behaviors (Overstreet et al., 1994). However, there are no data on serotonergic mechanisms in MCS patients.

A somewhat more complex neurotransmitter model proposes that the various neurochemical systems interact with one another and that abnormal behavioral states may arise from an alteration in one system which creates an imbalance in its interactions with others. For example Janowsky et al. (1972) proposed that depression and mania were the consequence of imbalances between the noradrenergic and cholinergic systems, with depression being associated with relative cholinergic overactivity and mania being associated with relative noradrenergic overactivity. An animal parallel to this observations was reported by Fibiger et al. (1970). This model can account for some of the effects observed in the FSL rats following administration of noncholinergic drugs. For

example, FSL rats are more sensitive to the hypothermic effects of dopamine agonists, but less sensitive to their sterotypy-inducing effects (Table 1; Crocker and Overstreet, 1991). Since dopaminergic and cholinergic systems work in parallel to regulate temperature but in opposition to regulate activity and stereotypy, an overactive cholinergic system could account for the findings with the dopamine agonists (See Overstreet, 1993). A similar argument could be made for chollinergic-serotonergic interactions as underlying depression and MCS.

Another type of mechanism which could underlie all MCS, depression and FSL rats is a change in second messenger rather than neurotransmitter functions. Several investigators have proposed that changes in G proteins, cyclic AMP or other second messenger systems may be involved in depression (Lesch and Manji, 1992; Avissar and Schreiber, 1993; Wachtel, 1989). Furthermore, it has been argued that the functional muscarinic responses in the FSL and FRL rats are too divergent to be accounted for by the relatively small differences noted in muscarinic receptors (Overstreet, 1993). This "downstream" hypothesis may more easily account for the pervasiveness of the chemical sensitivity described in MCS patients, which involves many classes of chemical compounds besides those having direct effects on neurotransmitter systems. Differences in second messengers could be hereditary or induced by exposure to chemical agents or by the effects of chemical agents on cholinergic or monoaminergic mechanisms. Further study of FSL rats, MCS patients, and depressed patients using diverse approaches is needed to obtain a greater understanding of the mechanisms that may underlie MCS.

## PRELIMINARY FINDINGS ON PYRIDOSTIGMINE

We propose that the characteristics of the animal model we have described are sufficiently analogous to MCS to warrant its use in testing hypotheses about the etiology and mechanisms of action involved in the syndrome. An example of the type of experimental protocols suggested by this review is the study of FSL and FRL rats after exposure to volatile solvents and other chemicals to which MCS patients report intolerance. This could be done with or without pre-existing exposure to cholinergic agents. If FSL rats do exhibit increased sensitivity to a wide variety of

chemical agents, then treatment approaches could be attempted, for example using antidepressant drugs. It should be emphasized that proposing antidepressant treatment does not presume that depression is the cause of MCS; indeed, quite the reverse might be true. For example, exposure to OPs might augment cholinergic sensitivity, leading to both MCS and depression. The possibility that increased cholinergic sensitivity might underlie both MCS and depression suggests further experiments in these patient groups. Questions which could be explored are whether there is a subset of depressed patients who report intolerance to varied substances and whether these same patients exhibit a greater sensitivity to cholinergic agents? A further question could be whether this subset of depressed patients would benefit from avoidance of certain drugs and environmental exposures. Finally, it would be of interest to know whether MCS patients have altered cholinergic responsivity, particularly in light of a recent study demonstrated that chronic fatigue syndrome, which is related to MCS, is associated with cholinergic supersensitivity (Chaudhuri et al., 1 997).

Another research direction that could be taken is to propose that individual differences in cholinergic sensitivity may have, in part, accounted for the varied responses of Gulf War participants to pyridostigmine and other agents. Given the large differences in cholinergic sensitivity between the FSL and FRL rats, we would predict substantial differences in responses to pyridostigmine in these animals. The remainder of this section will summarize the preliminary results of our findings.

FSL and FRL rats were selected from breeding colonies maintained at the University of North Carolina at Chapel Hill and randomly bred Sprague-Dawley (SD) rats (from which the FSL and FRL rats were originally derived) were obtained to act as a reference group. Both males and females were used. At about 70 days of age the rats were injected i.p. with sodium pentobarbital (35 mg/kg) to induce anesthesia for implanting the telemetry transmitters, which provided continuous monitoring of core body temperature, general activity, and, in some cases, heart rate.

After a one week period to allow recovery, the FSL, FRL and SD rats were adapted to the home cages for 24 hr and then injected s.c. with a mixture of peripherally acting methyl atropine (MA, 2.0 mg/kg) and oxotremorine (OXO, 0.2 mg/kg) to determine hypothermic responses. As can

be seen in Figure 1, the FSL rats exhibited the greatest hypothermic responses to OXO, as expected. However, the randomly bred SD rats were significantly more hypothermic than the FRL rats, suggesting that both lines have now diverged from control rats.

Approximately three days after the MA/OXO challenge, the rats were given pyridostigmine (PYR) bromide by gavage. The design called for four groups (vehicle and 4, 12, 36 mg/kg), but only the initial results from the two higher doses will be reported here. Temperature and activity were continuously recorded for 30 min after the PYR. The rats were then sacrificed by decapitation and blood removed and stored for the later analysis for cholinesterase activity and growth hormone levels. These assays are still in progress, so we will report on the physiological results in this communication; in addition, because the effects of PYR were not very striking, with little or no evidence of line differences, only the FSL and FRL data will be presented.

PYR had relatively few detectable effects on core body temperature at 12 mg/kg (Fig. 2A, 2B). There appeared to be a line difference in the females at 36 mg/kg (Fig. 3A), but neither the FSL nor the FRL rats exhibited any obvious hypothermia. In contrast, both male FSL and FRL rats exhibited modest but similar hypothermic responses to 36 mg/kg PYR (Fig. 3B). There were no detectable line differences in the effects of PYR on activity (data not shown).

These relatively small effects of PYR were not unexpected because it is a quaternary compound and does not normally get into the brain. However, Friedman et al. (1996) have shown that PYR can penetrate the blood-brain barrier in mice exposed to stressors, so it was thought that the FSL rats, which are more sensitive to stressors (See Overstreet, 1993; Overstreet et al., 1995), might exhibit a hypothermic response to PYR and the FRL rats would not. The fact that both male groups exhibit very similar small responses after 36 mg/kg PYR suggests that they both have intact blood-brain barriers. Experiments on the effects of pyridostigmine in the two lines after exposure to stressors are needed to clarify this issue.

As indicated above, the growth hormone assays are still in progress. We expect them to be quite revealing, because it has been well documented that PYR, despite its inability to penetrate the BBB, significantly increases growth hormone levels in both rats and humans (Martin et al., 1978;

Mazza et al., 1994). In fact, patients with a variety of ailments, such as depression, obsessive compulsive disorders, and chronic fatigue syndrome, exhibit abnormally responses to PYR (Chaudhuri et al., 1997; Ghigo et al., 1993; Lucey et al., 1993; O'Keane et al., 1992, 1994). Since some of these patient groups exhibit behavioral symptoms overlapping with or similar to those described in Gulf War veterans, it is possible that they too may exhibit abnormal responses, but no such study is available at yet. The FSL and FRL rats may thus represent animal analogs of patient and control groups, respectively, and can be useful in elucidating the mechanism of action of PYR.

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Table 1

Multiple Chemical Sensitivity in FSL Rats

A. Drug Classes to which FSL rats are more sensitive than FRL rats

| Drug Class | Compound | Responses |
|---|---|---|
| Anticholinesterase | DFP | Temperature/drinking |
| Anticholinesterase | Physostigmine | Temperature/activity |
| Muscarinic Agonist | Oxotremorine | Temperature/activity |
| Muscarinic Agonist | Pilocarpine | Temperature/activity |
| Muscarinic Agonist | Arecoline | Temperature/activity |
| Nicotinic Agonist | Nicotine | Temperature/activity |
| Dopamine D1/2 Agonist | Apomorphine | Temperature |
| Dopamine D2 Agonist | Quinpirole | Temperature |
| Dopamine D2 Antagonist | Raclopride | Catalepsy |
| 5-HT-1B Agonist | mCPP | Temperature/activity |
| 5-HT-1A Agonist | 8-OH-DPAT | Temperature |
| 5-HT-1A Agonist | Buspirone | Temperature |
| Benzodiazepine Agonist | Diazepam | Temperature/activity |
| Multiple (GABA, 5-HT) | Ethanol | Temperature |

Table 2

Comparison of Characteristics and Behavioral Features of

MCS Patients, FSL Rats and Depressed Patients

| MEASURE | MCS PATIENTS | FSL RATS | DEPRESSED PATIENTS |
|---|---|---|---|
| Weight | up or down | down | up or down |
| Appetite | up or down | down | up or down |
| Blood Pressure | up or down | ND | up or down |
| Food Craving | ++ | + | + |
| Sleep Disturbances | +++ | ++ | +++ |
| Loss of Drive | +++ | +++ | +++ |
| Reduced Activity | +++ | +++ | +++ |
| Cognitive Disturbance | +++ | +/- | +++ |
| Gender Ratios (F/M) | 4/1 | F>M | 2/1 |

ND = Not Determined

Table 3

Comparison of Drug Sensitivity in MCS Patients, FSL Rats and Depressed Patients

| COMPOUND | MCS PATIENTS | FSL RATS | DEPRESSED PATIENTS |
|---|---|---|---|
| Anticholinesterases | +++ | +++ | +++ |
| Solvents, etc. | +++ | ND | ND |
| Ethanol | +++ | ++ | +? |
| Nicotine | +++ | ++ | +? |
| Xanthines | +++ | ND | ND |
| Foods | +++ | ND | ND |

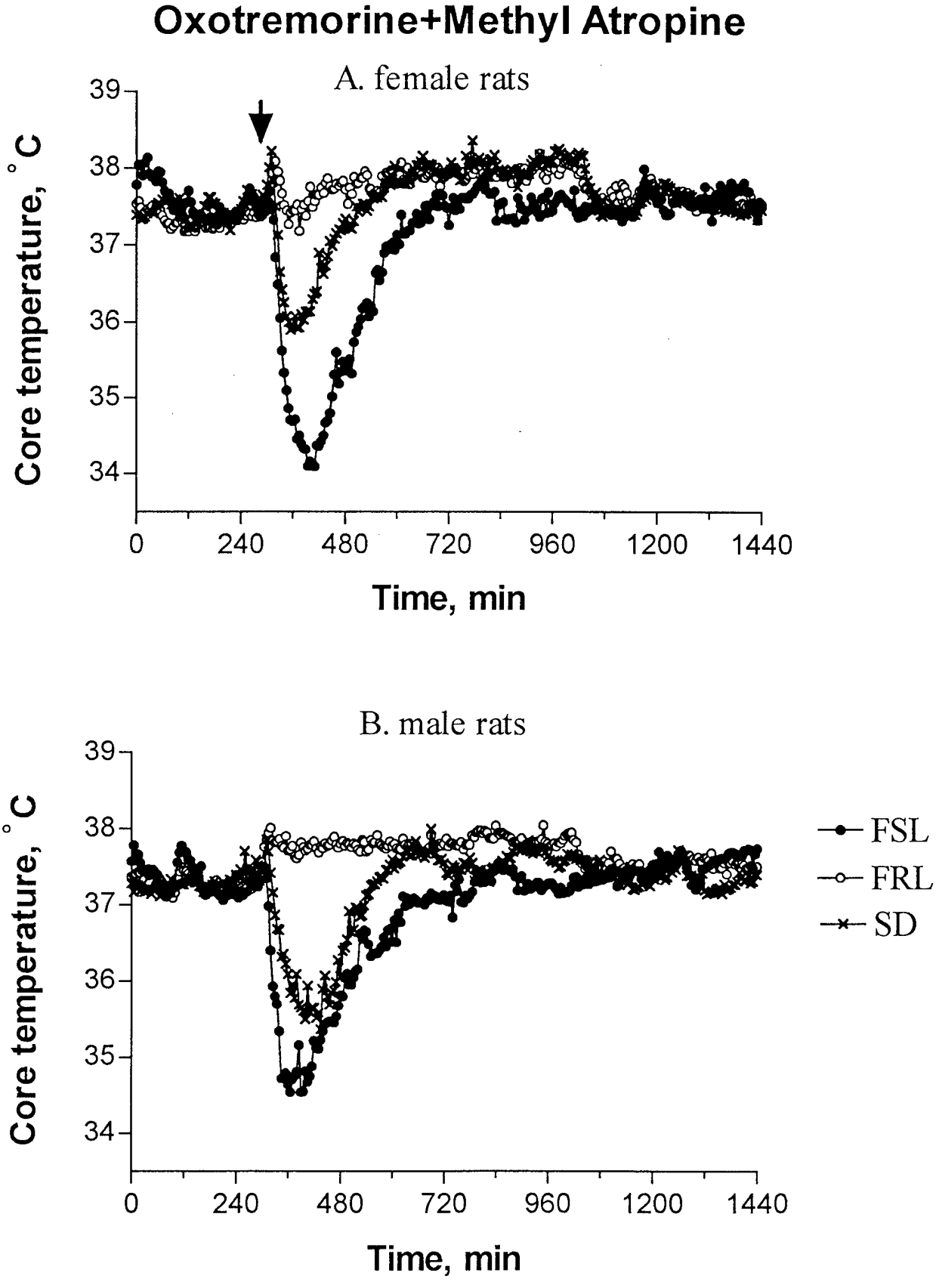
ND = Not Determined.

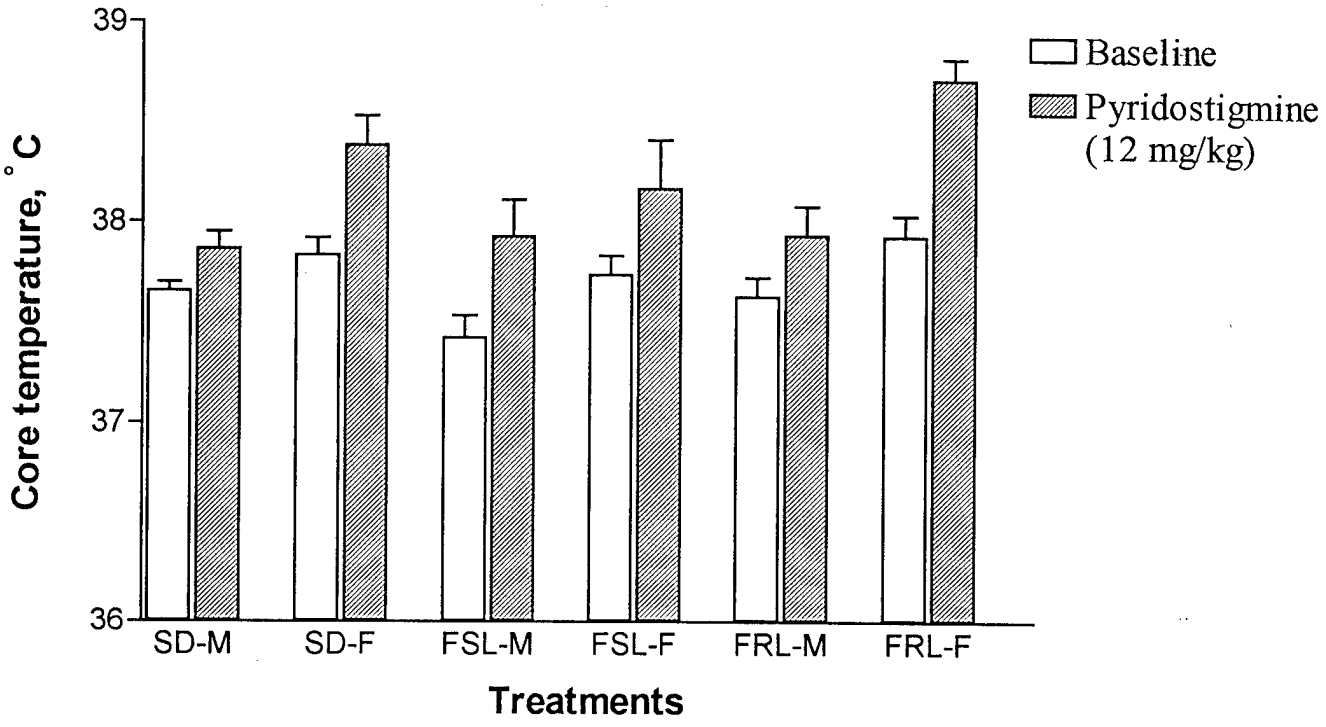
## FIGURE CAPTIONS

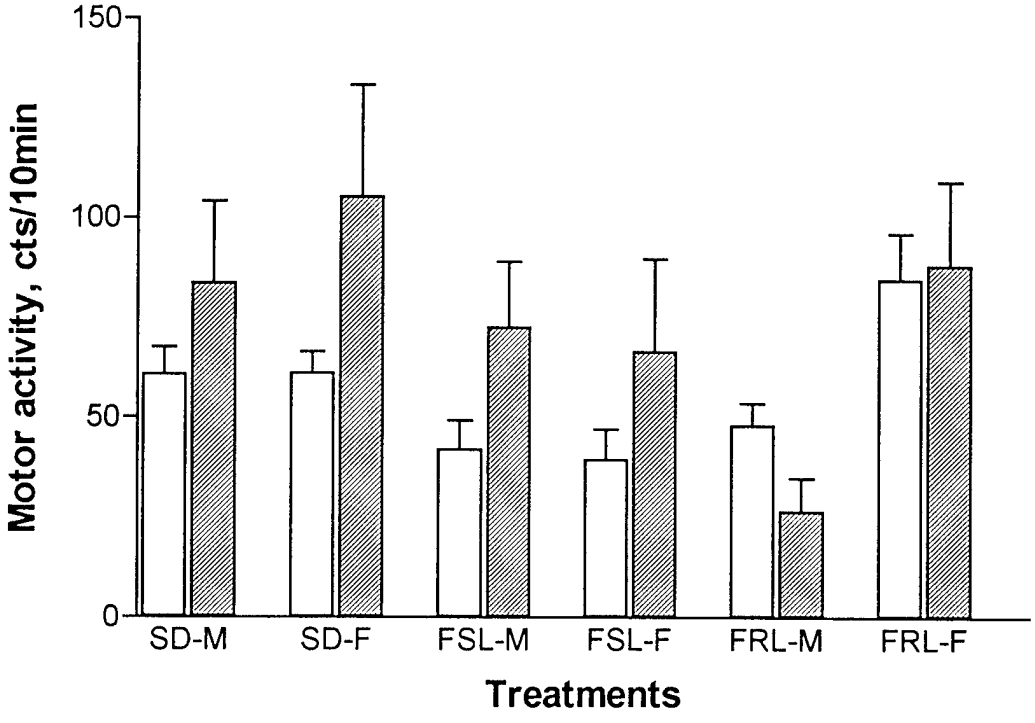
FIGURE 1. Hypothermic Effects of Oxotremorine in Telemetrically Monitored FSL, FRL and Sprague-Dawley rats. The results are the mean temperatures of 10 males and 10 females in each group. Note that the FSL rats exhibit the greatest peak decreases in temperature and the Sprague-Dawley rats have intermediate responses.

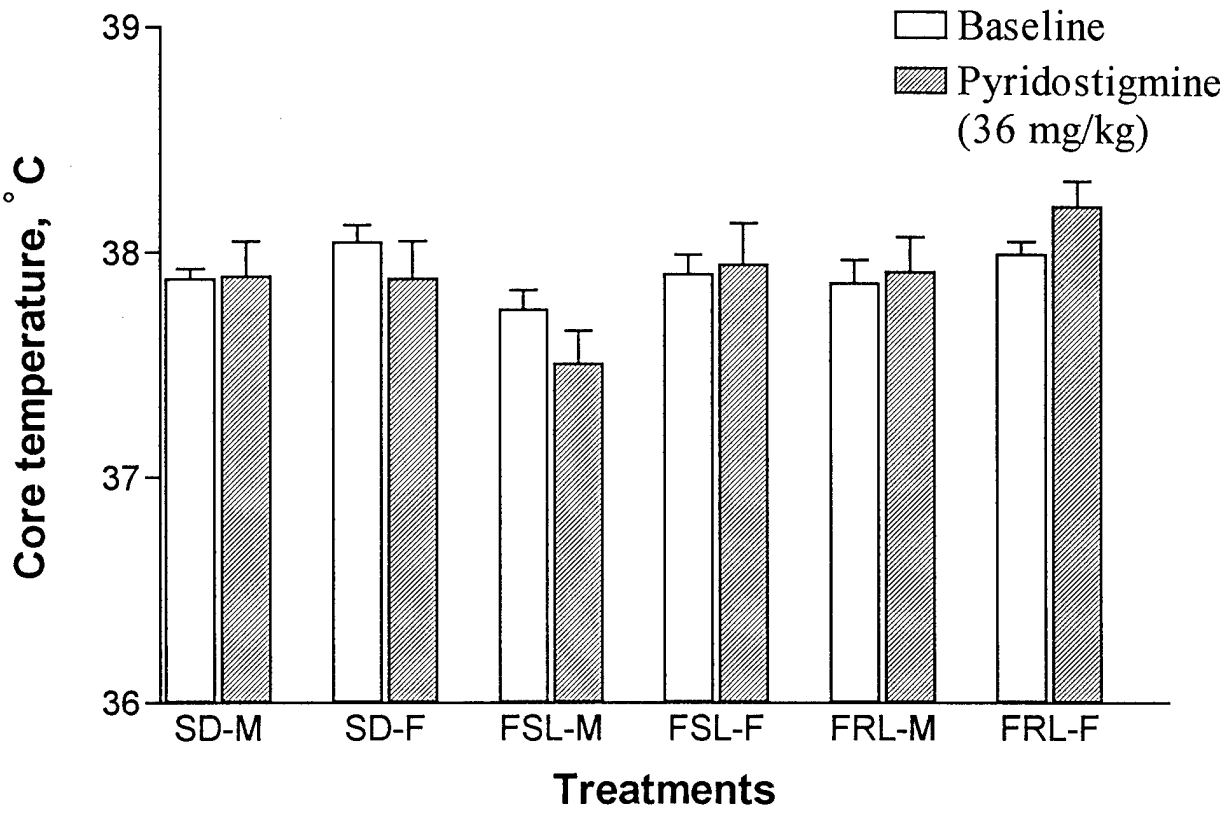
FIGURE 2. The Effects of Pyridostigmine (12 mg/kg, orally) on Core Body Temperature in Male (A) and Female (B) FSL and FRL rats. The results are the mean temperatures of 5 animals per group.

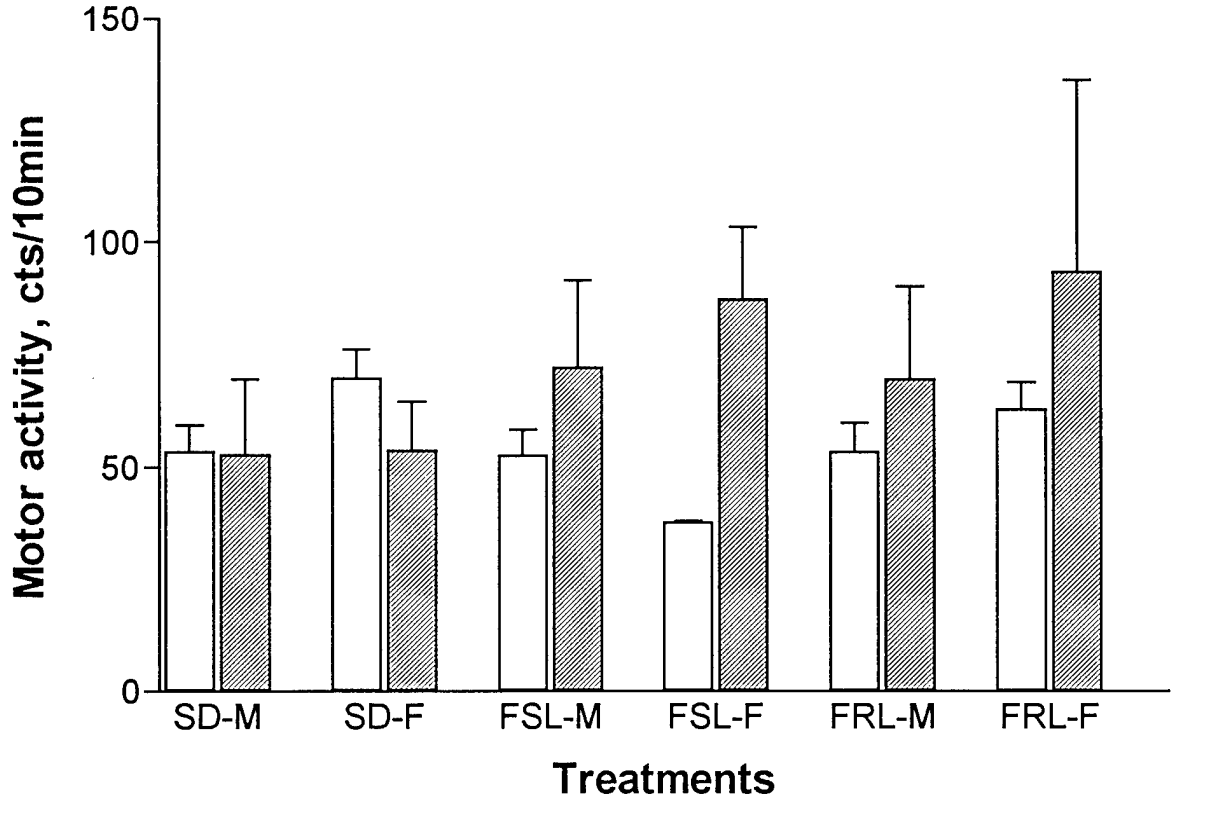
FIGURE 3. The Effects of Pyridostigmine (36 mg/kg, orally) on Core Body Temperature in Male (A) and Female (B) FSL and FRL rats. The results are the mean temperatures of 5 animals per group.


Oxotremorine+Methyl Atropine
A. female rats
Core temperature, °C
39
38
37
36
35
34
0
240
480
720
960
1200
1440
Time, min
B. male rats
Core temperature, °C
39
38
37
36
35
34
0
240
480
720
960
1200
1440
Time, min
FSL
FRL
SD


39
38
37
36
Core temperature, °C
Baseline
Pyridostigmine
(12 mg/kg)
SD-M
SD-F
FSL-M
FSL-F
FRL-M
FRL-F
Treatments


150
100
50
0
Motor activity, cts/10min
SD-M
SD-F
FSL-M
FSL-F
FRL-M
FRL-F
Treatments


Baseline
Pyridostigmine
(36 mg/kg)
Core temperature, °C
39
38
37
36
SD-M
SD-F
FSL-M
FSL-F
FRL-M
FRL-F
Treatments


Motor activity, cts/10min
150
100
50
0
SD-M
SD-F
FSL-M
FSL-F
FRL-M
FRL-F
Treatments

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# Cholinergic/Serotonergic Interactions in Hypothermia: Implications for Rat Models of Depression

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OVERSTREET, D. H., L. C. DAWS, G. D. SCHILLER, J. ORBACH AND D. S. JANOWSKY. *Cholinergic/serotonergic interactions in hypothermia: Implications for rat models of depression.* PHARMACOL BIOCHEM BEHAV **59**(4) 777–785, 1998.—This article reviews published reports and presents new evidence that support a number of commonalties between lines of rats selectively bred for differences in cholinergic (muscarinic) and serotonergic (5-$HT_{1A}$) sensitivity. The Flinders Sensitive Line (FSL) rat, a genetic animal model of depression derived for cholinergic supersensitivity, is more sensitive to both cholinergic and serotonergic agonists, and exhibits exaggerated immobility in the forced swim test relative to the control, Flinders Resistant Line (FRL), rat. Similar exaggerated responses are seen in a line of rats recently selected for increased sensitivity to the 5-$HT_{1A}$ agonist, 8-OH-DPAT (High DPAT Sensitive—HDS), relative to lines selectively bred for either low (Low DPAT Sensitive—LDS) or random (Random DPAT Sensitive—RDS) sensitivity to 8-OH-DPAT. For both the FSL and HDS rats, their exaggerated immobility in the forced swim test is reduced following chronic treatment with antidepressants. The present studies examined further the interaction between cholinergic and serotonergic systems in the above lines. Supersensitive hypothermic responses to 8-OH-DPAT were observed very early (postnatal day 18) in FSL rats, suggesting that both muscarinic and serotonergic supersensitivity are inherent characteristics of these rats. Scopolamine, a muscarinic antagonist, completely blocked the hypothermic effects of the muscarinic agonist oxotremorine in FSL and FRL rats, but had no effect on the hypothermic responses to 8-OH-DPAT, suggesting an independence of muscarinic and 5-$HT_{1A}$ systems. On the other hand, genetic selection of genetically heterogeneous rats for differential hypothermic responses to the muscarinic agonist oxotremorine were accompanied by differential hypothermic responses to 8-OH-DPAT, suggesting an interaction between muscarinic and 5-$HT_{1A}$ systems. Overall, these studies argue for an inherent interaction between muscarinic and 5-$HT_{1A}$ systems, which probably occurs beyond the postsynaptic receptors, possibly at the level of G proteins.
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Serotonin 8-OH-DPAT Muscarinic Oxotremorine Ontogeny FSL rat Depression Core body temperature

ALTHOUGH the underlying neurochemical components of depressive disorders are still largely unknown, it has been postulated that an interaction of, or dysbalance between, two or more neurotransmitter systems is involved [e.g., (6,26)]. Although other combinations have been put forward since Janowsky and colleagues originally proposed the cholinergic–adrenergic hypothesis of depression in 1972 (26), a hypothesis involving cholinergic–serotonergic interactions in depression has received relatively little attention to date (42,44). The present article reviews the evidence for an interaction be-

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tween serotonergic and cholinergic systems in two rat models of depression and describes new data supporting such an interaction in the regulation of temperature.

The involvement of the serotonergic system in depression and related affective disorders is now well recognized. Reports of alterations in serotonin (5-HT) receptors and/or receptor function in depressed individuals (2,6,8,31,67), downregulation of the 5-HT receptor/second messenger systems by clinically effective antidepressants [e.g. (4,18,33)], and 5-$HT_{1A}$ receptor agonists being potentially effective antidepressants (12,34) are just a small part of the evidence that has implicated 5-HT in the pathogenesis and treatment of depression. Furthermore, serotonergic "supersensitivity" has recently been reported in depressed individuals with, for example, increased 5-$HT_2$ receptor function, measured as increased phosphoinositide hydrolysis in the platelets of depressed humans, occurring after 5-$HT_2$ agonist administration (36).

In addition, central cholinergic neurotransmitter mechanisms have long been implicated in the pathogenesis of depressive disorders (25,26,28). It is well recognized that individuals with depressive disorders are more sensitive to the behavioral (i.e., depression-inducing) and physiological (e.g., elevation of adrenocortical hormones and growth hormone, induction of REM sleep) effects of muscarinic agonists than are normal controls [e.g. (7,27,37,52,60).

With respect to cholinegic and serotonergic interactions, it has been reported that brain regions that are integral in the regulation of mood and cognition, such as the cerebral cortex and hippocampus, are rich in muscarinic receptors (mAChR) (35) and receive a dense serotonergic innervation as well (64). Pharmacological studies suggest that both systems are involved in the regulation of passive avoidance behavior (51), which might relate to depression in humans (39). Biochemical (1,19) manipulations suggest that 5-HT release may be regulated by muscarinic receptors (19), whose plasticity is dependent upon the integrity of the serotonergic system (1). Thus, not only are the cholinergic and serotonergic systems anatomically related to each other, but they also interact in such a way that a dysbalance of one system may lead to a functional deficit in the other. The etiology of affective disorders may, therefore, be attributable to a dysbalance between these neurotransmitter systems, with, for example, cholinergic overactivity predisposing to depression but subsequent alterations in serotonergic function actually inducing the depressive episodes.

Potential genetic animal models of depression have been developed by selective breeding for differential responses to muscarinic and serotonergic agonists, respectively (45–47), and the implications of these models for a cholinergic/serotonergic interaction hypothesis of depression will be the focus of the present communication. The Flinders Sensitive Line (FSL) rats represent a cholinergic model of depression (39,46). These rats were originally selectively bred to be more sensitive to anticholinesterases than the control Flinders Resistant Line (FRL) rats (41,54). However, FSL rats are also more sensitive to the behavioral and physiological effects of directly acting muscarinic agonists (39,40,46). Furthermore, FSL rats also are more sensitive to a variety of serotonergic drugs, including those that target 5-$HT_{1A}$ and 5-$HT_2$ receptors (42,56,66). Preliminary experiments have reported a positive correlation between increased behavioral sensitivity and increased 5-HT receptor number in the FSL rats (56).

More recently, randomly bred, genetically heterogeneous rats were used to selectively breed for differential hypothermic responses to the selective 5-$HT_{1A}$ receptor agonist, 8-OH-DPAT. The line that became more sensitive to 8-OH-DPAT (the High DPAT Sensitive—HDS line) exhibited a number of similarities to the FSL rats. In addition to their supersensitive responses to 5-$HT_{1A}$ agonists, they exhibited exaggerated immobility in the forced swim test (46,49), and this immobility could be counteracted by chronic treatment with antidepressant drugs (24,49,58). Both HDS and FSL rats also exhibit higher consumption of sweet solutions (13,47,50), and both appear to have elevated numbers of cortical 5-$HT_{1A}$ receptor binding sites (30,47,56). Thus, there are several intriguing parallels between the HDS rats, selectively bred for increased 5-$HT_{1A}$ sensitivity, and the FSL rats, selectively bred for increased muscarinic sensitivity.

What is particularly intriguing about these genetic animal models of depression is that although the FSL and HDS rats were selectively bred for increased hypothermic responses to oxotremorine (66) and 8-OH-DPAT (44), respectively, they exhibit similar behavioral profiles and antidepressant-like responses to clinically effective antidepressant agents (24,46,47). Table 1 summarizes these similarities among depressed individuals and the FSL and HDS rats. Note that despite several similarities, neither the HDS nor the FSL rats resemble depressed individuals in serotonergic sensitivity. The predominant approach has been to challenge depressed and control individuals with serotonergic agonists and measure specific hormones, and the most common finding is for the depressed individuals to display a blunted response (31). Hormonal responses to serotonergic challenges have not been studied in the FSL or HDS rats, but these lines are supersensitive to the hypothermic effects of 8-OH-DPAT, as mentioned above (see Table 1). Therefore, these rat models do not mimic all aspects of depressed individuals. Nevertheless, they are innately more immobile in the forced swim test and are less immobile following chronic treatment with antidepressants (Table 1).

In contrast to what appeared to occur in the FSL rats (42,66), it initially appeared that selection for differential 5-$HT_{1A}$ sensitivity was not accompanied by a parallel increase in muscarinic sensitivity as such (45). This observation, when coupled with the results of an interbreeding study that indicated little correlation between 5-$HT_{1A}$ and muscarinic responses in genetically heterogeneous rats (44), suggested that the serotonergic and cholinergic systems were independently regulated. In the present set of experiments we sought to obtain data that would confirm or call into question the postulated independence of, or interaction between, the serotonergic and cholinergic systems, using drug-induced hypothermia as the index variable.

TABLE 1

SIMILARITIES AMONG DEPRESSED INDIVIDUALS AND FSL AND HDS RATS

| Feature/Measure | Depressed Individuals | FSL Rats | HDS Rats |
|---|---|---|---|
| Increased cholinergic sensitivity | Yes | Yes | Yes |
| *Increased 5-$HT_{1A}$ sensitivity* | *No* | *Yes* | *Yes* |
| *Decreased locomotor activity* | *Yes* | *Yes* | *No* |
| Increased REM sleep | Yes | Yes | N.D. |
| High sweet intake craving | Yes | Yes | Yes |
| Immobility after stress | Yes | Yes | Yes |
| Antidepressant response | Yes | Yes | Yes |

Areas where there is a lack of agreement are highlighted in italics.

The three approaches used were: 1) a developmental profile of 8-OH-DPAT sensitivity in the FSL and FRL rats to determine if it is similar to the developmental profile observed in FSL rats for the muscarinic agonist, oxotremorine (11); 2) a classical pharmacological blockade study of the ability of scopolamine to counteract the hypothermic effects of oxotremorine and 8-OH-DPAT; 3) a short-term selective breeding study focusing on the development of oxotremorine sensitivity, with a parallel examination of changes in 5-$HT_{1A}$ (i.e., 8-OH-DPAT) sensitivity.

## METHOD AND RESULTS

### *Experiment 1. Developmental Profile of 8-OH-DPAT Sensitivity in FSL and FRL Rats*

Developmental profiles have shown that the FSL rats are more sensitive to the hypothermic effects of oxotremorine than are their control counterparts, the FRL rats, as early as 2 weeks postnatal, the earliest age of practical testing (10,11). Using this developmental approach, the present study aimed to compile a profile for hypothermic sensitivity to the 5-$HT_{1A}$ receptor agonist, 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) in FSL and FRL rats of different ages.

*Animals.* Male and female FSL and FRL rats aged 15, 20, 25, 31, and 60 days of age were selected from the 47th generation of FSL and FRL colonies being maintained at the Flinders University of South Australia. Body weights ranged from a mean of 29.1 ± 0.8 g ($n$ = 36 pooled genders) at 15 days of age to 267 ± 8 g for males ($n$ = 42) and 188 ± 4 g for females ($n$ = 37) at 60 days of age. Data for 150- and 250-day-old rats were derived from the 43rd and 46th generations, respectively. At 250 days of age males weighed 507 ± 12 g ($n$ = 23) and females, 309 ± 8 g ($n$ = 21). Until the time of weaning (30 days of age) all rats were housed with respective dams and removed only during test sessions. After this time they were housed in groups of six in large metal cages with free access to food and water. The colony room was maintained at 22 ± 1°C and 50% humidity, under a 12 L:12 D cycle. The number of rats used for each test ranged between 3 and 10 of each gender. All experiments were conducted between 0800 and 1300 h. This experiment was approved by the Institutional Animal Care Committee of Flinders University.

*Core body temperature recording.* Core body temperature was recorded by inserting a lubricated thermocouple probe (Eirelec 5000 hand-held thermometer), 1 to 5 cm into the rectum (i.e., the distance being proportional to the age and size of the rat). Temperature was recorded to the nearest 0.1°C and was stable within 1 min after insertion of the probe. Baseline temperatures were always obtained within the 2 h preceding drug challenge. The data are typically expressed as mean ± standard error of the mean (SEM) deviations from baseline where each animal served as its own control.

*Procedure.* Rats were weighed and baseline core body temperatures obtained on the morning of the drug challenge tests. Animals were quasi-randomly divided into two groups and subcutaneously injected with either isotonic saline (SAL) or 8-OH-DPAT (0.1 mg/kg). Core body temperature was recorded 30 min later. The two groups were selected in such a way that there was an equal representation from each litter in the two treatment groups. The dose of 8-OH-DPAT was selected on the basis of dose–response curves for 8-OH-DPAT-induced hypothermia in adult FSL and FRL rats (57); with 0.1 mg/kg producing near maximal hypothermia and clearly delineated sensitivity differences between the two lines. To minimize the possibility of drug tolerance occurring, the treatment groups were alternated so that there was a minimum of 10 days between exposure to either 8-OH-DPAT or SAL. As an additional check for tolerance development and for further clarification of the developmental profile, a subset of rats from each litter were left drug "naive." These rats were given 8-OH-DPAT once only at selected ages (17, 18, 24, and 30 days of age). 8-OH-DPAT hydrobromide was obtained from Research Biochemicals Incorporated (Natick, MA). The dose refers to the weight of the salt and was freshly prepared on each challenge day by dissolving in isotonic saline and kept on ice to avoid degradation.

*Statistical analysis.* The data were subjected to multiple analysis of variance using the statistical package SPSS-X on a UNIX system mainframe computer. Where there were no significant gender differences, data for male and females were pooled. Line, age, and treatment were the factors tested. The probability level for significance was set at $p < 0.05$. Prior to any inferential statistics analyses, data were tested for homogeneity of variance using Bartlett-Box F and Cochran's C-tests. Both confirmed homogeneity of variance.

*Results.* Within each line there were no significant gender differences in change in core body temperature after 8-OH-DPAT; therefore, the data for males and females were pooled. The results depicted in Fig. 1 highlight the pronounced differences in sensitivity between the FSL and FRL rats with respect to 8-OH-DPAT–induced hypothermia. The FSL rats displayed a significantly greater 8-OH-DPAT–induced hypothermic effect than the FRL rats at all ages tested with the exception of hypothermia at 15 days of age, where the lines were not different. This yielded a significant main effect of line, $F(1, 276) = 184.64$, $p < 0.001$. The magnitude of this difference varied with age, being maximal at 18 and 31 days of age, and yielded a significant main effect of age, $F(9, 276) = 43.88$, $p < 0.001$. Furthermore, an interaction effect between line and age was established (Fig. 1). FSL, relative to FRL rats, became more sensitive to the hypothermic effect of 8-OH-DPAT with age [line × age, $F(9, 276) = 5.87$, $p < 0.001$].

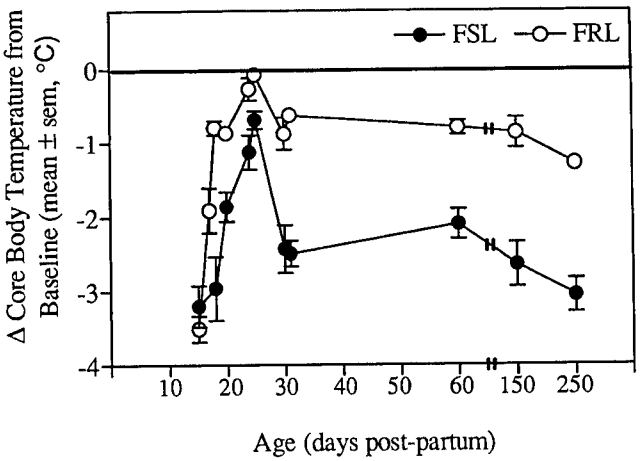


FIG. 1. Age-dependent changes in mean core body temperature after 0.1 mg/kg (SC) 8-OH DPAT. Data for male and female rats were pooled because no significant gender differences were established with respect to drug-induced change in core body temperature. There were 6 to 20 rats per group. Each animal served as its own control and change in temperature is with respect to normal baseline core body temperature.

8-OH-DPAT "naive" rats, depicted at 17, 18, 24, and 30 days of age in Fig. 1, did not deviate from the general developmental pattern observed in rats that received 8-OH-DPAT on three separate occasions at 10-day intervals.

Saline-injected controls exhibited minor fluctuations about a mean of 0°C change in core body temperature over all ages tested (data not shown), with the extreme ranges being from −0.4 to +0.2°C.

*Experiment 2: Pharmacological Blockade of 8-OH- DPAT–Induced Hypothermia*

This experiment explored the possibility that 5-$HT_{1A}$ sensitivity, as shown by hypothermia, is caused by muscarinic sensitivity in the Flinders Line rats due to serotonergic neurons synapsing on cholinergic neurons, which transmit to the heat loss pathways. If this model of cholinergic neurons being the final common path to hypothermia is correct, then scopolamine, a centrally acting muscarinic antagonist, should block or partially counteract the hypothermic effect of 8-OH-DPAT, the 5-$HT_{1A}$ receptor agonist, as well as that of oxotremorine, the muscarinic agonist. Experiments were conducted in adult FSL and FRL rats to investigate this hypothesis.

*Animals.* Male and female FSL and FRL rats were selected from the 48th generation of the breeding colonies maintained at Flinders University. The rats were between 75–80 days old at the beginning of the study and weighed approximately 350 g (for males) or 205 g (for females). The rats were housed and maintained as described above. This experiment was approved by the Institutional Animal Care Committee of Flinders University.

*Procedure.* The rats were randomly divided into eight treatment groups so that there was an even representation of litter mates in each group (7–16 rats per group). The groups received either isotonic saline (four groups) or scopolamine (0.2 mg/kg, four groups) pretreatments followed by saline, oxotremorine (0.19 mg/kg), or 8-OH-DPAT (0.1 or 0.5 mg/kg) 15 min later. Scopolamine hydrochloride and oxotremorine sesquifumarate were obtained from Sigma (St. Louis, MO), and 8-OH-DPAT was obtained from Research Biochemicals Incorporated (Natick, MA); doses refer to the salts for the drugs. Core body temperatures were recorded at baseline, when the animals were weighed, and at 30 min after the final injection. All injections were given SC in 1 ml/kg.

*Statistical analyses.* Results are expressed as mean ± SEM changes in °C from the corresponding baseline measures. The data were subjected to two-way ANOVAs, with line and treatment as the main factors. Post hoc analyses were performed using Scheffe's multiple contrast tests. Where appropriate, *t*-tests were performed to test the significance of selected pairs of data.

*Results.* The effects of scopolamine pretreatment on oxotremorine- and 8-OH-DPAT–induced hypothermia in the FSL and FRL rats are illustrated in Fig. 2. Two-way ANOVA indicated highly significant treatment ($F = 80.7$, $p < 0.001$) and line ($F = 142.8$, $p < 0.001$) effects. The saline vehicle produced a small hyperthermic response, while scopolamine slightly reduced temperature in the FSL rats and increased it in the FRL rats (Fig. 2A). Scopolamine significantly counteracted the decrease in body temperature induced by oxotremorine in both lines, as expected (Fig. 2A). In contrast, the hypothermia induced by either dose of 8-OH-DPAT was only slightly affected by scopolamine pretreatment (Fig. 2B). Thus, these findings indicate that scopolamine selectively blocks hypothermia induced by the muscarinic receptor agonist, oxotremorine.

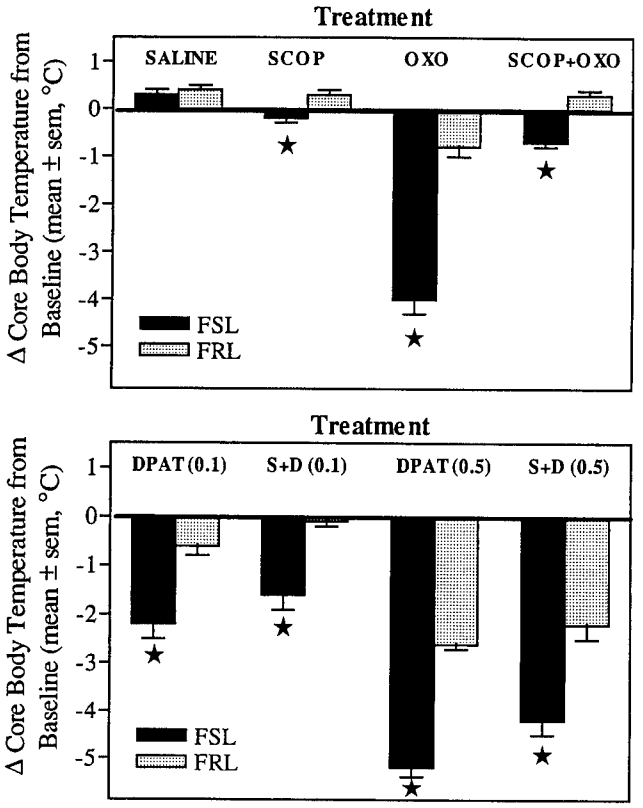


FIG. 2. Scopolamine blockade of hypothermia induced by oxotremorine but not 8-OH-DPAT. Scopolamine (0.2 mg/kg) or saline vehicle was given SC 15 min prior to the administration of saline vehicle, 8-OH-DPAT (0.1 or 0.5 mg/kg), or oxotremorine (0.19 mg/kg). Data represent the mean ± SEM changes in °C from baseline for 7–16 rats.

*Experiment 3: Genetic Selection for Differential Hypothermic Responses to Oxotremorine*

When the serotonergic sensitivity differences were first discovered in the FSL and FRL rats (66), 13 generations of selection for muscarinic sensitivity had occurred. Consequently, the apparent association between muscarinic and 5-$HT_{1A}$ sensitivity may have occurred by chance and not by a genetic correlation. The fact that muscarinic and 5-$HT_{1A}$ sensitivities are not significantly correlated in the previously mentioned intercross experiments between the FSL and FRL rats (43) would appear to support the lack of close genetic association between the cholinergic and serotonergic sensitivity. However, we have found dramatic differences in muscarinic sensitivity to oxotremorine in later generations of the randomly bred genetically heterogeneous rats that were selectively bred for differential hypothermic responses to 8-OH-DPAT, despite only small differences in the earlier generations (43,45). These studies thus provide mixed support for the hypothesis of a genetic association between muscarinic and 5-$HT_{1A}$ sensitivities.

The present experiment sought to clarify this relationship by using randomly bred genetically heterogeneous rats to conduct a short-term selective breeding study in which serotonergic and cholinergic sensitivity were simultaneously evaluated in rats bred for differences in oxotremorine sensitivity.

*Animals.* The animals were selected from a genetically heterogeneous (N/Nih) breeding colony that was established in the Center for Alcohol Studies at the University of North Carolina (45). Since obtaining breeding stock from NIH, these rats have been maintained by breeding 10 pairs per generation, with no matings occurring between close relatives. Litter size averages 10–12 rats. The rats were housed in standard housing conditions under a reversed 12 L:12 D cycle, with lights off between 1000 and 2200 h. This experiment was approved by the Institutional Animal Care Committee of the University of North Carolina.

*Procedure.* The study began with an oxotremorine challenge at weaning (28–32 days of age). The rats were marked, weighed, and baseline temperatures were recorded with a rectal thermocouple probe attached to a Sensortek digital thermometer. They were then injected SC with a drug mixture containing oxotremorine (0.2 mg/kg) and atropine methyl nitrate (2 mg/kg). Core temperatures were recorded 30 min later and rats were selected for breeding according to their hypothermic responses. The male and female rat from each litter that exhibited the greatest decrease in temperature were used to establish the High Oxotremorine Sensitivity (HOS) group; those that exhibited the smallest decrease in temperature were used to establish the Low Oxotremorine Sensitivity (LOS) group.

Animals were paired for mating so that there were no close relatives. In the first generation progeny, the same procedures as described above were carried out at weaning: handling, weighing, recording of baseline temperature, injection of oxotremorine/methyl atropine mixture, recording of temperature at 30 min. Again, the most affected male and female were used to continue the HOS line and the least affected male and female were used to continue the LOS line.

The same procedure was followed once again for the second generation progeny, with one addition. Approximately 5 days after the oxotremorine challenge, the rats were given a single 0.5 mg/kg SC injection of 8-OH-DPAT, and core temperature was recorded 45 min later, as is typically done in the 8-OH-DPAT–selected rats (45,47).

To provide a reference group, data from the 7th generation of the HDS and LDS rats, selected for their differential hypothermic responses to 8-OH-DPAT, were included. The rats were first given 8-OH-DPAT (0.5 mg/kg, SC) at weaning and their temperatures recorded 45 min later. Then, approximately 5 days later, they were challenged with a mixture of oxotremorine and methyl atropine, as described above, and temperatures recorded 30 min later. Finally, in addition to the LOS and HOS groups, selectively bred for differences in oxotremorine-induced hypothermia, and the HDS and LDS groups, selectively bred for differences in 8-OH-DPAT–induced hypothermia, a group of randomly bred genetically heterogeneous (RDS) rats were included.

Oxotremorine sesquifumarate and atropine methyl nitrate were obtained from Sigma (St. Louis, MO) and 8-OH-DPAT was obtained from Research Biochemicals Incorporated (Natick, MA). Doses refer to the salts of the respective drugs.

*Statistical analyses.* The data were analyzed by one-way ANOVAs, with follow-up Newman–Keuls tests.

*Results.* Hypothermia induced by oxotremorine or 8-OH-DPAT was studied in similarly maintained animals from the 7th generation of selection of the 8-OH-DPAT–selected lines (HDS and LDS) and the 2nd generation of selection of the oxotremorine-selected lines (HOS and LOS). There were highly significant ($F = 57.18$, $p < 0.0001$) differences among the groups. In Fig. 3, it can be seen that there appeared to be

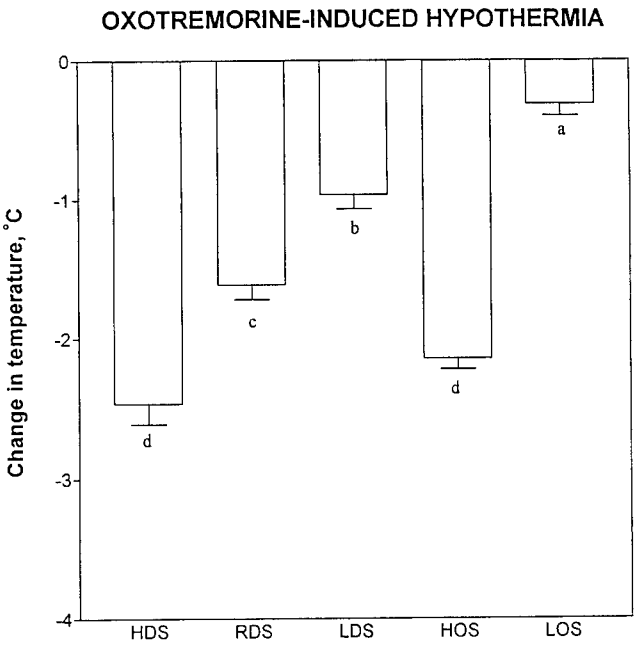


FIG. 3. Hypothermia induced by oxotremorine in rats selectively bred for differential hypothermic responses to oxotremorine or 8-OH-DPAT. A mixture of oxotremorine (0.2 mg/kg) and methyl atropine (2 mg/kg) was administered 30 min prior to the recording of core temperature by a rectal thermistor probe. The data represent the mean ± SEM changes in °C from baseline for 17–36 male and female rats. Groups with different letters are significantly different, $p < 0.01$, Newman–Keuls test.

rapid selection for the differential hypothermic response to oxotremorine, as the HOS rats exhibited a significantly greater hypothermic response than the LOS rats, with the randomly bred (RDS) rats intermediate. This figure also shows that there are significant differences in the hypothermic response to oxotremorine in the HDS and LDS rats, selectively bred for differential hypothermic responses to 8-OH-DPAT. The HDS rats were more sensitive to oxotremorine.

The converse data, illustrated in Fig. 4, present a rather similar picture. There are large and significant differences in hypothermic responses to 8-OH-DPAT in the lines selectively bred for differential responses to this agent (HDS and LDS), but there are also significant differences between the HOS and LOS lines, selectively bred for differences in oxotremorine-induced hypothermia ($F = 100.61$, $p < 0.001$). The HOS line is more sensitive to the hypothermic effects induced by 8-OH-DPAT. As for oxotremorine, the randomly bred RDS rats are intermediate between the HDS and LDS rats.

## GENERAL DISCUSSION

The present findings, utilizing three diverse approaches, argue for an interaction between the cholinergic and serotonergic systems in these rat models of depression. However, the hypothesis that simple changes in 5-$HT_{1A}$ or muscarinic receptors can account for the present findings cannot be supported, and alternative mechanisms must be considered.

Experiment 1 demonstrated that both FSL and FRL rats exhibited a hypothermic response to 8-OH-DPAT at the earliest age tested, suggesting that the 5-$HT_{1A}$ system is already

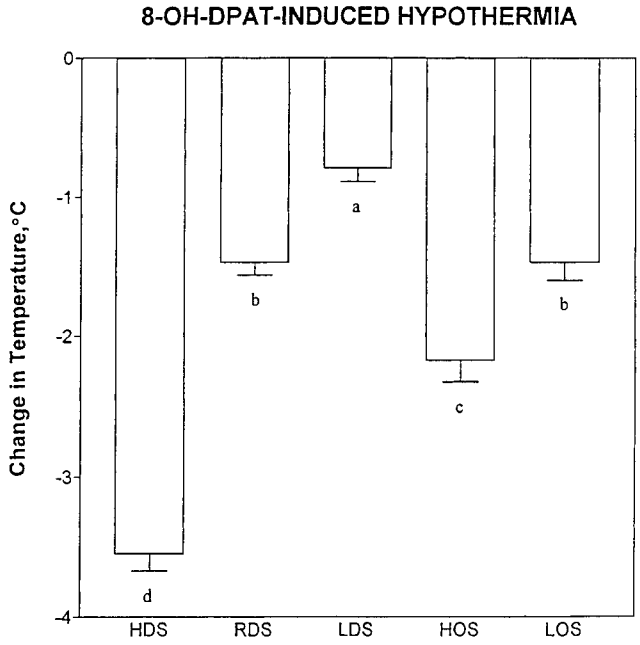


FIG. 4. Hypothermia induced by 8-OH-DPAT in rats selectively bred for differential hypothermic responses to oxotremorine or 8-OH-DPAT. 8-OH-DPAT (0.5 mg/kg) was administered 45 min prior to the recording of core temperature by a rectal thermistor probe. The data represent the mean ± SEM changes in °C from baseline for 20–31 male and female rats. Groups with different letters are significantly different, $p < 0.01$, Newman–Keuls test.

functional at 15 days of age. The ability of 8-OH-DPAT to exert an effect early in life is in good agreement with the literature (14,29,53,62,63). The present results are further supported by the observation that saline did not result in a significant change in core body temperature from baseline. The ability of saline-treated pups to maintain a constant body temperature when separated from their respective dams for extended periods of time indicates that the capacity to thermoregulate efficiently is present at 15 days of age and thus is not a confounding variable.

Because the FRL rats were considered control rats for the developmental (i.e., ontogenetic) study, it is appropriate to first compare the data derived from FRL rats with those in the literature for randomly bred Sprague–Dawley rats. There is a paucity of literature regarding 8-OH-DPAT–induced hypothermia in juvenile rats. However, the decrease in core body temperature (approx. −1.0°C) exhibited by adult FRL rats is comparable to reports where data have been obtained under similar conditions [e.g., (17,21,22)]. The greater hypothermia (−2.0 to 3.5°C) observed in rats aged 15–17 days is perhaps not surprising, because sensitivity to various serotonergic and cholinergic drugs, quantified using a variety of behavioral measures, have been reported to alter with age (53,61). For example, the 5-HT antagonist, metergoline, inhibited suckling in 3–4- and 7–8-day-old rat pups but had little influence on suckling behavior in older 21–24-day-old pups (53). Receptor number and/or affinity, coupling to second messengers, and integration of neurotransmitter systems often undergo dynamic changes during the first 2–3 postnatal weeks. These changes may not solely serve as precursors for the adult neurotransmitter systems but may also be related to the mediation of behaviors essential to the young pup (53). In summary, the FRL rats responded to a pharmacological manipulation of the serotonergic system that closely resembled that described in the literature. Any gross deviation from this ontogenetic profile exhibited by the FSL rats is, therefore, very likely to be a trait of these selectively bred rats.

Comparison of the developmental profiles for FSL and FRL rats revealed marked differences in their sensitivity to 8-OH-DPAT. FSL rats older than 15 days were supersensitive to the hypothermic effect of 8-OH-DPAT. Thus, 5-$HT_{1A}$ supersensitivity, like muscarinic supersensitivity (10), appears to be an inherent characteristic of the FSL rats. Figure 1 illustrates the virtually parallel development of sensitivity to 8-OH-DPAT–induced hypothermia in FSL compared to FRL rats. The FSL rats, aside from exhibiting greater hypothermia, did not deviate from the ontogenetic pattern of the FRL rats. Both lines were least sensitive to the hypothermic effect of 8-OH-DPAT at 25 days of age. Sensitivity to 8-OH-DPAT then increased quite rapidly, and levels of adult responsivity were reached at 30 days of age. This pattern was not attributable to the emergence of tolerance to 8-OH-DPAT, because age-matched 8-OH-DPAT naive rats exhibited a hypothermia that fell within the bounds of the developmental profile derived from the main experimental group that received 8-OH-DPAT on three separate occasions. One functional implication of such change in sensitivity during development, as described earlier, may be the need for the suppression of neonate behaviors as adult behaviors emerge (55). The underlying neurochemical basis(es) for this period of relative insensitivity cannot be determined from these data alone, and so we can only speculate. However, it is noteworthy that the ontogenetic profile for sensitivity to the muscarinic agonist oxotremorine was similar in shape but the period of relative insensitivity occurred earlier (18 days of age) than for 8-OH-DPAT in FSL rats [(11); see Fig. 5].

Transient periods of altered sensitivity to drugs do not appear to be unique to a single neurotransmitter system and are probably attributable to the dynamic changes that take place during synaptogenesis in the early postnatal weeks. For example, the efficacy of receptor–second messenger coupling (5) and/or expression of genes involved in the manufacture of the various enzymes and receptors that combine to form the functional adult neural network undergo marked changes during the early period in development (19,22,23). The 5-$HT_{1A}$ receptor gene is one such example. A high rate of expression is observed during a limited period in fetal life and again at 18 days of age, after which time the level of gene expression falls quite markedly (20). It is tempting to postulate that after 25 days of age, the FSL rats undergo a further period of 5-HT receptor gene overexpression, which may, at least in part, explain their "adult" supersensitivity to 8 OH-DPAT. Indeed, this is an appealing hypothesis, because preliminary receptor binding studies have indicated an approximately 20% increase in 5-HTlA receptor number in adult FSL compared to FRL rats (56) and in the HDS compared to LDS rats (30,47).

Although the neurochemical and/or metabolic changes that occur early in development are complex, it is clear that the FSL and FRL rats are different in their response to both 8-OH-DPAT and oxotremorine [(10,11); Fig. 5]. The most striking difference is the enhanced sensitivity of FSL rats to the hypothermic effect of these agonists. A more subtle difference, highlighted in Fig. 5, is the ontogenetic time frame for the periods of relative insensitivity to the hypothermic effect of 8-OH-DPAT and oxotremorine. As discussed earlier, both FSL and FRL rats are least sensitive to 8-OH-DPAT at 25

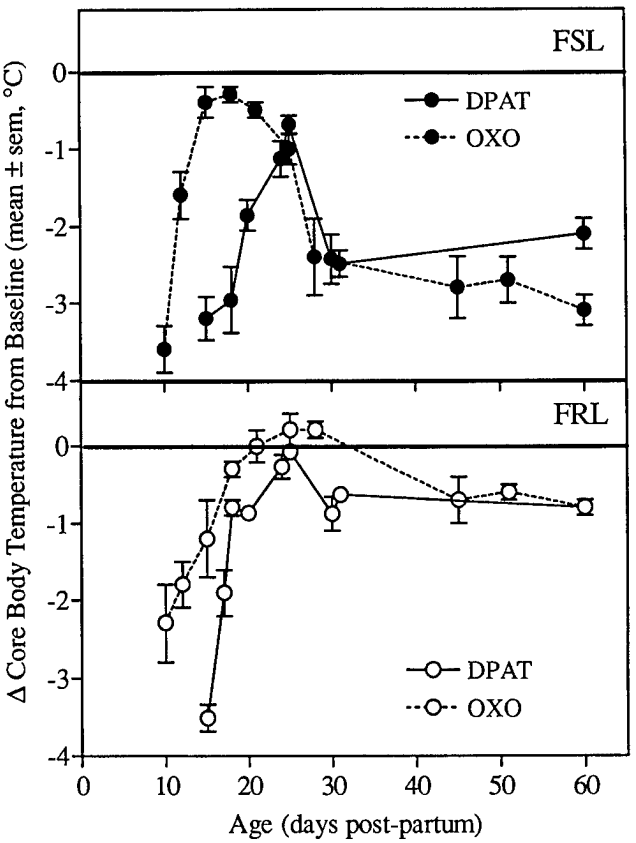


FIG. 5. A comparison of age-dependent changes in mean core body temperature after 0.1 mg/kg (SC) 8-OH-DPAT or 0.25 μmol/kg (SC) oxotremorine. Data for 8-OH-DPAT is duplicated from Fig. 1 and data for oxotremorine is modified from Daws and Overstreet (submitted). Data for male and female rats (within a line) were pooled because no significant gender differences were established with respect to drug-induced change in core body temperature. There were 5 to 20 rats per group. Each animal served as its own control and change in temperature is with respect to normal baseline core body temperature. Associated standard error of the means were not greater than 0.4°C and have been omitted for the sake of clarity. Solid symbols = FSL rats, open symbols = FRL rats; circles = 8-OH-DPAT, squares = oxotremorine.

days of age. The FRL rats are also least sensitive to oxotremorine-induced hypothermia at 25 days of age. This contrasts with the FSL rats, where the greatest insensitivity to oxotremorine-induced hypothermia occurs at 18 days of age. Thus, there are inherent differences not only in the sensitivity of these rat lines to muscarinic and serotonergic agonists, but also in the nature of their developmental profiles. Recent cross-breeding studies using the FSL and FRL rats suggest that muscarinic sensitivity is under the influence of additive and dominance genetic factors, whereas serotonergic sensitivity appears to be influenced by solely additive genetic factors (43,45). Together with the findings of the developmental studies, it appears that both cholinergic and serotonergic systems are instrumental in determining the altered phenotype of the FSL rats.

There is a growing body of literature regarding the genetics of affective disorder in humans (15), and recent studies have shown serotonergic dysfunction in prepubertal major depression patients (55) as well as cholinergic supersensitivity in children at risk for depression (59). The early emergence of serotonergic supersensitivity in the FSL rats provides yet another characteristic to add to the growing suite of parallels between the FSL rats and human depressives (39). Thus, the FSL animal model of conjoint cholinergic/serotonergic supersensitivity may well be heuristic in understanding the neurochemical causes of depressive illness, particularly with respect to a cholinergic–serotonergic balance hypothesis.

Unfortunately, there have been no studies to date that have examined cholinergic or serotonergic drugs on temperature regulation in humans despite its relative noninvasiveness. Instead, human studies have more often focused on changes in neuroendocrine or sleep measures after challenge with serotonergic or cholinergic agents (9,28,31). Also, none of these studies have used both serotonergic and cholinergic probes in the same group of subjects, so it is impossible to assess the value of the cholinergic serotonergic balance hypothesis at this time. The studies on the rat models presented here argue strongly for the necessity of such parallel studies in humans.

Experiment 2 demonstrated that scopolamine blocked the hypothermic responses induced by the muscarinic agonist oxotremorine but not the 5-$HT_{1A}$ agonist 8-OH-DPAT (Fig. 2). Therefore, the serotonergic system probably is not linked in series with the cholinergic system in inducing hypothermia. There is other evidence from selectively bred rat lines that supersensitive hypothermic responses may not be dependent exclusively on changes in muscarinic or 5-$HT_{1A}$ receptors. The supersensitive muscarinic response can be seen very early (Fig. 5), but the muscarinic receptor elevations in the hypothalamus do not appear until 60 days of age (10). Despite the large differences in hypothermic responses to 8-OH-DPAT in the HDS and LDS lines, there are no differences in hypothalamic 5-$HT_{1A}$ receptors in these lines (30). Thus, the close developmental profiles of oxotremorine and 8-OH DPAT sensitivity and the parallel changes occurring during selective breeding must be accounted for by mechanisms other than simple changes in receptors.

However, the mechanism must be closely related to both cholinergic and serotonergic systems because, as Experiment 3 demonstrates, there were parallel changes in both oxotremorine- and 8-OH-DPAT–induced hypothermic responses when animals were selectively bred for differences in oxotremorine sensitivity (Fig. 3). This study provided, therefore, confirmatory evidence for the association of 5-$HT_{1A}$ and muscarinic supersensitivity in the FSL rats (42,56,57,64). This experiment also demonstrated that the lines selectively bred for differential hypothermic responses to 8-OH-DPAT, the HDS and LDS rats, are now also differentially sensitive to the muscarinic agonist, oxotremorine (Fig. 4). These parallel changes in muscarinic and 5-$HT_{1A}$ sensitivity during selective breeding for either muscarinic or 5-$HT_{1A}$ sensitivity argue strongly for a common underlying mechanism.

One potential mechanism that could account for the above observation is an alteration in G proteins or in some other aspect of the second-messenger systems. According to various biochemical and molecular studies, both muscarinic M2 receptors and 5-$HT_{1A}$ receptors interact with a Gi protein which contributes to the inhibition of cyclic AMP (38). In contrast, the muscarinic M1 receptor and the 5-$HT_2$A receptor are positively linked to the phosphatidyl inositol second-messenger system (38). It is not clear at present whether the hypothermic effects of oxotremorine or OH-DPAT are mediated through a Gi protein. However, if they were and these proteins changed as a consequence of selective breeding, then the parallel changes in 5-$HT_{1A}$ and muscarinic sensitivity could be explained.

Furthermore, there is also considerable interest in the possibility that G proteins may be involved in the etiology and phenomenology of depression in humans (3,48), and that alterations in G protein function may accompany chronic treatment with antidepressant drugs (3,32,33). Thus, it is possible that both FSL and HDS rats exhibit exaggerated immobility in the forced swim test and other behavioral analogs of depression because selective breeding for the increased hypothermic responses to their respective drugs has resulted in a similar change in G protein function. An investigation of this hypothesis in the FSL and HDS models of depression may, in turn, help clarify the mechanisms underlying human depression, in particular, the growing body of evidence implicating altered G protein function in affective disorders (3,32,33,48).

This article would not do justice to the impressive literature on depressive disorders if it did not conclude with this cautionary note. Although almost all of the available evidence accumulated to date is consistent with cholinergic supersensitivity in depressive disorders (28), there is a wealth of information suggesting an association of serotonergic subsensitivity with affective disorders, and not serotonergic supersensitivity as indicated above. This serotonergic subsensitivity is most commonly observed as a blunted hormonal response to serotonergic drugs, such as serotonin reuptake inhibitors, in depressed individuals [e.g. (16,65)]. However, a recent review article has indicated that the findings with directly acting 5-HT receptor agonists are much less consistent with respect to hormonal subsensitivity, and has suggested that the apparent subsensitivity can be explained by a reduction in the release of 5-HT, rather than any change in 5-HT receptors (9). At present, there are no data on hormone levels in the FSL and HDS rats after challenges with serotonergic agents, so it is not possible to indicate how closely these animal models resemble depressed individuals with respect to hormonal subsensitivity. Similarly, as indicated above, there is no information on the effects of cholinergic and serotonergic drugs on temperature regulation in humans. Clearly, further studies must be performed before the serotonergic/cholinergic balance hypothesis can be accepted.

ACKNOWLEDGEMENTS

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# LINKS BETWEEN MULTIPLE CHEMICAL SENSITIVITY AND ASTHMA IN A RAT MODEL OF CHOLINERGIC HYPERSENSITIVITY: A BRIEF REVIEW

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## Abstract

Individuals with multiple chemical sensitivity (MCS) also commonly report symptoms of asthma, but, as far as we have been able to determine, no one has yet suggested that an abnormal cholinergic system may provide the link between asthma and MCS. The present brief review provides evidence for such a link by summarizing recent findings in a genetic animal model of cholinergic hyperresponsiveness. The Flinders Sensitive Line (FSL) rats were developed by selective breeding for increased responses to an anticholinesterase agent similar to commonly used organophosphate pesticides. Relative to their control line, the Flinders Resistant Line (FRL) rats, the FSL rats are more sensitive to drugs that stimulate acetylcholine receptors, alcohol, diazepam, and drugs that have a selective effect on dopamine or serotonin receptors. These findings raise the possibility that the FSL rat may resemble individuals with MCS. Hyperresponsiveness of the airways is a hallmark of asthma. The procedure known as whole body plethysmography, where breathing can be monitored in freely moving animals, was employed to study the FSL and FRL rats. The FSL rats exhibited a greater index of bronchoconstriction than the FRL rats in response to both a cholinergic agonist and an allergen challenge. Thus, the FSL rats are more sensitive both to a variety of drugs unrelated to the cholinergic system and to cholinergic- and allergen-induced bronchoconstriction. An abnormal cholinergic system may therefore contribute to both MCS and asthma.

## Introduction

Multiple chemical sensitivity (MCS) and asthma are commonly reported conditions; for example, a recent survey has indicated that allergies and/or chemical sensitivity are reported by over 50% of the population, with 16.9% reporting simultaneous allergy and chemical sensitivity (Meggs et al., 1996). Other investigators have noted that asthma is frequently found in those individuals with multiple chemical sensitivity (e.g., Levy, 1997; Ross, 1997; Ziem and McTanney, 1997). The overlap in the prevalence of these two conditions suggests that there might be some underlying common mechanism. The present brief review considers the hypothesis that a hyperresponsive cholinergic system might provide a link between asthma and MCS by considering evidence recently obtained in a genetic animal model of cholinergic hyperresponsiveness (Djuric et al., 1998; Overstreet et al., 1996).

The cholinergic nervous system, which utilizes acetylcholine at its nerve terminals, is very pervasive (Barnes, 1992; Mesulam, 1995). Not only does it innervate voluntary skeletal muscles (acting upon nicotinic receptors) and involuntary smooth muscles and the heart (acting upon muscarinic receptors), but it also send fibers into many regions of the brain from basal forebrain and midbrain nuclei (Mesulam, 1995). Stimulation of cholinergic pathways via pesticides and other agents may produce a variety of peripheral and central symptoms which are reminiscent of the complaints of people suffering from asthma and/or MCS, including respiratory, gastrointestinal, and CNS symptoms. Indeed, a patient's hyperresponsiveness to the cholinergic agonist methacholine is commonly used to confirm a diagnosis of asthma. Therefore, the pervasiveness of the cholinergic system makes it a likely candidate to be involved in the multi-organ complaints frequently reported by individuals with MCS, including difficulties in concentration, irritable bowel, and asthma (Levy, 1997; Ross, 1997).

The hypothesis that a hyperresponsive cholinergic system is a link between asthma and MCS will be examined in this communication by briefly reviewing some findings from a genetic animal model of cholinergic hypersensitivity. Specifically, the increased sensitivity of this animal model to a variety of drugs acting upon multiple neurotransmitter systems and its

increased sensitivity to breathing difficulties induced by an allergen will be summarized (See Djuric et al., 1998; Overstreet et al., 1996, for details).

## An Animal Model of Cholinergic Hypersensitivity

The Flinders Sensitive Line (FSL) rat was developed by selective breeding for increased responses to the anticholinesterase agent, diisopropyl fluorophosphate (Overstreet et al., 1979; Russell et al., 1982). The Flinders Resistant Line (FRL) rat was bred in a parallel manner for reduced responses and typically does not differ from randomly bred control rats (Overstreet et al., 1979: Russell et al., 1982). The FSL rat also is more sensitive to directly acting muscarinic and nicotinic agonists (Overstreet et al., 1995, 1996) and have elevated brain muscarinic and nicotinic receptors in selected brain regions (Overstreet et al., 1984; Tizabi et al., 1999). The cholinergic hyperresponsiveness of the FSL rat appears to be an innate trait, because is can be observed as early as a functional response can be recorded (Daws et al., 1991; Daws and Overstreet, 1999). As illustrated in Table 1, the FSL rats clearly have increased cholinergic sensitivity relative to the FRL rats. Both peripheral and central cholinergic responses appear to be enhanced (Djuric et al., 1995, 1998; Overstreet et al., 1996), although only CNS muscarinic and nicotinic receptors have been measured to date. We do not know whether the peripheral hyperresponsiveness in the FSL rats is a consequence of increased number of muscarinic receptors or due to changes in second messengers.

Insert Table 1 about here

## Multiple Chemical Sensitivity (MCS)

Evidence supporting the FSL rat as a model for MCS has been recently reviewed in some detail (Overstreet et al., 1996), so only a few key points will be made here. Behaviorally, MCS patients and FSL rats share a number of features, including fatigue, sleep disturbances, and reduced activity (See Overstreet et al., 1996, for a fuller description).

Clinical observations suggest that MCS may be initiated by acute or chronic exposure to diverse chemical agents. The FSL rats exhibit increased sensitivity to anticholinesterase agents and muscarinic agonists (Daws et al., 1991; Overstreet, 1986; Overstreet and Russell, 1982; Overstreet et al., 1992a,b; Schiller et al., 1988). Notably, there have also been several reports of increased sensitivity to anticholinesterases in MCS patients (Cone and Sult, 1992; Miller and Mitzel, 1995; Rosenthal and Cameron, 1991). At present there are no published data for MCS patients regarding sensitivity to direct cholinergic agonists in particular, but such agents are among those which many MCS patients say they cannot tolerate.

As illustrated in Table 2, the FSL rats are also more sensitive to the hypothermic effects of serotonergic agents. (Wallis et al., 1988, Overstreet et al., 1992, 1994). As yet, there are no data on the effects of selective serotonergic agents in MCS patients, so the similarity between the FSL rats and MCS patients for this parameter cannot be evaluated at present.

The reductions in body temperature and in locomotor activity induced by the beta noradrenergic agonist salbutamol were similar in the FSL and FRL rats (Overstreet et al., 1989). Similarly, the reductions in body temperature and in operant responding for water reward induced by the alpha noradrenergic agonist clonidine were also similar in the FSL and FRL rats (Overstreet, 1989). Thus, the FSL and FRL rats do not differ in response to every drug; there do not appear to be any marked differences in behaviorally represented noradrenergic function between FSL and FRL rats.

The FSL rats are supersensitive to the hypothermic (Crocker and Overstreet, 1991) and aggression-promoting (Pucilowski et al., 1991) effects of apomorphine, a mixed D1/D2 agonist, and quinpirole, a selective D2 agonist. On the other hand, the FSL rats are subsensitive to the stereotypy-inducing effects of apomorphine and quinpirole at similar doses where supersensitivity to the hypothermic effects were seen (Crocker and Overstreet, 1991). In addition, no evidence for differences in dopamine D2 receptors between FSL and FRL rats could be detected (Crocker and Overstreet, 1991). Consequently, it was argued that the opposite changes in sensitivity in the various functions could be related to the way the cholinergic and

dopaminergic systems interact to modulate those functions. Both cholinergic and dopaminergic stimulation promote hypothermic and aggressive responses (Cox et al., 1980; Pucilowski, 1987; Ray et al., 1989), but cholinergic stimulation has opposite effects to dopaminergic stimulation in the modulation of activity and stereotypy (Fibiger et al., 1970; Klemm, 1989).

Insert Table 2 about here

In addition to the above drugs which interact selectively with specific neurotransmitter receptors, the FSL and FRL rats are differentially sensitive to the effects of several other pharmacological agents, as summarized in Table 2. However, as with the case of dopamine agonists, the differential effects are observed only for some actions of the drugs, not for all. For example, ethanol induces a greater hypothermia in the FSL rats, but not a greater intoxication (Overstreet et al., 1990b). Similarly, diazepam produces greater behavioral suppressant effects in the FSL rats (Pepe et al., 1988), but the anxiolytic effects of diazepam in the two lines are comparable (Schiller et al., 1991).

In summary, it is quite clear that the FSL rat is more sensitive to a variety of chemical agents in addition to the anticholinesterase for which they were selectively bred. In this regard, the FSL rat is, in part, analogous to MCS patients who become more sensitive to a range of agents following exposure to organophosphate anticholinesterases (Miller and Mitzel, 1995). However, the reactions to the rats following exposure to solvents and other chemicals to which MCS patients are exquisitely sensitive have yet to be tested. Similarly, the reactions of MCS patients to serotonergic agents, to which FSL rats are also hyperresponsive have not been tested.

## Experimental Asthma

The FSL and FRL rats were selectively bred for differential responses to an anticholinesterase that has both central and peripheral effects. However, much of the early work with these rats focussed on changes in the brain and responses to centrally acting drugs (see Overstreet et al., 1995, 1996, for reviews). An early report of the FSL rats being more sensitive to carbachol, a peripherally acting muscarinic agonist (Overstreet and Russell, 1982) suggested

that peripheral cholinergic supersensitivity might also exist in the FSL rats. Collaborations with Canadian colleagues interested in the potential involvement of cholinergic mechanisms in conditions such as irritable bowel and asthma were initiated in the early 1990's.

In the first study, isolated intestinal strips from FSL rats were found to be more responsive to the muscarinic agonist bethanechol than were strips from the FRL rats (Djuric et al., 1995). In this study, the indices of systemic and intestinal allergy were also investigated. Following sensitization to ovalbumin (OA) rats of both lines were challenged in vivo with 3 mg OA or saline. The FSL rats were more susceptible to allergy than the FRL rats as evidence by more pronounced mast cell degranulation, more intense hypothermic reaction, higher hematocrit values, and increases in the transport tone and permeability of isolated small intestinal tissues (Djuric et al., 1995). Since there was no difference between the two lines in levels of circulating IgE antibodies, we concluded that other factors, neuroendocrine, are responsible for the greater susceptibility of the FSL rat.

The study above suggested that the hyperresponsiveness of intestinal tissues to cholinergic agonists in the FSL rats might be a contributory factor to their greater response to the antigen challenge. To determine whether the peripheral cholinergic hypersensitivity is pervasive in the FSL rats we turned our attention to another model system: experimental asthma. The details of this study have recently been published (Djuric et al., 1998), so a summary of the findings will be reported here.

The first experiment sought to determine whether there were differences in the FSL and FRL rats to challenge with the cholinergic agonist methacholine. The methacholine inhalation test is the most frequently used procedure to evaluate airway responsiveness. A whole body plethysmographic technique and computerized pulmonary analyzer (Buxco Electronics, Sharon, CT) was used to evaluate airway responsiveness to methacholine. With increasing concentration of methacholine, there was a parallel increase in the bronchoconstriction, as evidenced by enhanced pause of Penh, and the decrease in breathing rate. At the highest concentration used (256 mg/ml), methacholine induced a decrease in breaths per min from a baseline of 150 to 125

in the FSL rats, while there was a much smaller change in the FRL rats, from 150 to 145 (See Djuric et al., 1998, for other values). Thus, FSL rats were confirmed to be hyperresponsive to methacholine, just as are asthma patients.

The second experiment established that the FSL rats were also more sensitive to the allergen-induced changes in airway responsiveness. Following exposure to nebulized antigen (5% OA) for 5 min , the FSL rats exhibited very dramatic bronchoconstriction and the ensuing inflammation of the airways that was assessed by counting inflammatory cells (eosinophils and neutrophils) obtained from bronchoalveolar lavage (BAL) fluids 24 hr later. The BAL fluids from the FSL rats had greater numbers of white blood cells and higher relative proportions of neutrophils and eosinophils, indicating a more pronounced inflammation of the airways (Djuric, et al, 1998).

A third experiment compared both Flinders lines with their parent strain, the Sprague-Dawley rats, since with only two groups in the comparison, it was not clear which group differed from the normal population. The results confirmed that only the FSL rats differed from the population norm. There were no differences between the FRL and Sprague-Dawley rats; only the FSL rat exhibited abnormal, heightened responses to the antigen challenge. The heightened responses, including both the increases in bronchoconstriction and breathing difficulty appeared shortly after exposure to the antigen, while the increased numbers of inflammatory cells obtained from BAL fluids was observed 24 hr later (Djuric et al., 1998). These findings clearly establish that the FSL rat is exhibiting abnormal, heightened responses to the antigen challenge. Therefore, the possibility of a hyperresponsive cholinergic system underlying both asthma and MCS is supported.

## Mechanisms underlying Cholinergic hyperresponsiveness

The papers reviewed in this communication are consistent with the hypothesis that cholinergic hyperresponsiveness may contribute both to asthma and to MCS. However, in a major review of the neurotransmitter pathways in the airways, Barnes (1992) points out the

importance of the cholinergic pathways, both pre- and post-ganglionic, but tends to discount changes in the postsynaptic component as underlying asthma. The present findings argue for a reconsideration of these views. It is possible that the postsynaptic cholinergic hyperresponsiveness is particularly relevant to those cases of asthma which have a genetic contribution.

The mechanisms underlying the postsynaptic cholinergic hyperresponsiveness of the FSL rat have not been elucidated. Certainly, there are elevated muscarinic receptors in the brain of the FSL rats (Overstreet et al., 1984). However, the increased responsiveness of the FSL to the hypothermic effects of oxotremorine is observed prior to there being any evidence of elevated muscarinic receptors (Daws et al., 1991; Overstreet et al., 1998; Daws and Overstreet, 1999). The peripheral hyperresponsiveness of FSL rats has been studied only recently, so receptor-binding studies have yet to be performed. Since muscarinic receptors are key in the airways (Barnes, 1992), studies of these receptors could be informative.

Another potential mechanism which might underlie the cholinergic hyperresponsiveness is an alteration in some component of the second messenger cascade which occurs following the interaction of acetylcholine with the muscarinic receptor. Changes in G protein function, cyclic AMP, and/or phosphatidyl inositol are just a few of the candidates. The attractiveness of this hypothesis is that it might also account for the increased sensitivity of the FSL rats to some of the noncholinergic drugs. For example, serotonergic agonists that stimulate the 5-HT1A receptor subtype may utilize the same second messenger pathway as cholinergic agonists (See Overstreet et al., 1998). Determining the mechanisms underlying the hyperresponsiveness of the FSL rats to drug-induced bronchoconstriction may be useful in better understanding both asthma and MCS.

## Conclusion

The FSL rats are clearly more sensitive to cholinergic agonists, both centrally and peripherally (Djuric et al., 1995, 1998; Overstreet et al., 1995, 1998). They are also more

sensitive to the effects of a variety of other drugs influencing other neurotransmitter systems, suggesting responses similar to MCS patients (Miller and Mitzel, 1995; Overstreet et al., 1996). However, as indicated above, solvents, perfumes and other exotic odors that have been known to trigger responses in patients with MCS have not been tested in the Flinders rats. Since these substances do not have known mechanisms of action, it is not clear what predictions one might make. The whole body plethysmography technique may be suitably adapted to test these compounds. Since the FSL rats are more sensitive to the bronchoconstriction induced by both the cholinergic agonist methacholine and the relatively nonspecific antigen ovalbumin, it is likely that they may also be hyperresponsive to these odors as well.

Allergies have been commonly reported in depressed patients as well as those with MCS (Marshall, 1993; Meggs et al., 1996). Depression is also a commonly reported symptom of MCS patients and the similarities between depression, MCS and behavioral symptoms in the FSL rats have recently been reviewed (Overstreet et al., 1996). Others have suggested that a cholinergic hyperresponsiveness may be the common link between depression and allergies (Marshall, 1993). Thus, a group of conditions with overlapping symptoms, including depressive disorders e.g. Janowsky et al., 1994), irritable bowel syndrome (White et al., 1991), asthma (Djuric et al., 1998), chronic fatigue syndrome (Chaudhuri et al., 1997), and MCS (Overstreet et al., 1996) may be influenced by a pathological, hyperresponsive cholinergic system.

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Table 1

Hyperresponsiveness of FSL Rats to Cholinergic Drugs

| Compound | Mechanism of Action | Responses |
|---|---|---|
| DFP | Anticholinesterase | Hypothermia/FSL>FRL<br>Water Intake<br>Locomotor Activity |
| Physostigmine | Anticholinesterase | Hypothermia/FSL>FRL |
| Oxotremorine | Muscarinic Agonist | Hypothermia/FSL>FRL<br>Locomotor Activity<br>Bar Pressing for Water |
| Pilocarpine | Muscarinic Agonist | Hypothermia/FSL>FRL |
| Arecoline | Muscarinic Agonist | Hypothermia/FSL>FRL<br>Locomotor Activity<br>Bar Pressing for Water |
| Nicotine | Nicotinic Agonist | Hypothermia/FSL>FRL<br>Bar Pressing for Water |
| Bethanecholine | Muscarinic Agonist | Intestinal Transport/FSL>FRL |
| Methacholine | Muscarinic Agonist | Breathing Difficulty/FSL >FRL |

Adapted from Overstreet et al. (1996) and Djuric et al. (1995, 1998).

Table 2

Differences in Responses between FSL and FRL Rats to Noncholinergic Drugs

| Compound | Mechanism of Action | Response |
|---|---|---|
| Apomorphine | Dopamine D1/2 Agonist | Hypothermia/FSL>FRL |
| Quinpirole | Dopamine D2 Agonist | Hypothermia/FSL>FRL |
| Raclopride | Dopamine D2 Antagonist | Catalepsy/FSL>FRL |
| mCPP | 5-HT-1B Agonist | Hypothermia/FSL>FRL |
| 8-OH-DPAT | 5-HT-1A Agonist | Hypothermia/FSL>FRL |
| Buspirone | 5-HT-1A Agonist | Hypothermia/FSL>FRL |
| Diazepam | Benzodiazepine Agonist | Hypothermia/FSL>FRL |
| Ethanol | Multiple (GABA, 5-HT) | Hypothermia/FSL>FRL |
| Apomorphine | Dopamine D1/2 Agonist | Stereotypy/FSL<FRL |
| Quinpirole | Dopamine D2 Agonist | Stereotypy/FSL<FRL |
| MK-801 | NMDA Antagonist | Hyperthermia/FSL<FRL |

Adapted from Overstreet et al. (1996).

# ORGANOPHOSPHATE PESTICIDES, CHOLINERGIC FUNCTION AND COGNITIVE PERFORMANCE IN ADVANCED AGE

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**Abstract**. Overstreet DH. Organophosphate pesticides, cholinergic function, and cognitive performance in advanced age. The present communication will address the question of whether older individuals/animals are at greater risk to the cognitive impairment induced by exposure to organophosphates (OPs). There is considerable evidence for a decline in cholinergic indices (choline acetyltransferase, acetylcholinesterase, and muscarinic acetylcholine receptors) with aging. Whether these decreases lead to a greater or smaller response to OPs will depend on the relative rate of change in the specific indices. Some studies have shown that aged animals are more sensitive to cholinergic (muscarinic) agonists, even though the receptors are reduced. These findings suggest that aged individuals may be more sensitive to OPs. There is also much evidence documenting the decline in cognitive function in aged animals, a process which is accentuated in Alzheimer's disease and which has been attributed, in part, to the decline in cholinergic indices. Several studies have reported an improvement in cognitive functioning in aged animals/humans following treatment with acetylcholinesterase inhibitors. Thus, it is possible that the cognitive impairment, which occurs during aging, may be partially counteracted during treatment with certain cholinesterase inhibitors (both OPs and nonOPs). Whether the cognitive impairment reported by younger individuals some time after exposure to OPs would be greater in the elderly cannot be predicted on the basis of the available evidence. However, the findings in an animal model of dietary-induced cholinergic hypofunction developed by Roger Russell and colleagues may shed light on this question.

Running Head: Organophosphates and Aged Individuals

**Key Words: Aged Individuals; Cognitive Function; Organophosphates (OPs); Acetylcholinesterase; Choline Acetyltransferase; Muscarinic Receptors; Cholinergic Sensitivity; OP Tolerance.**

## INTRODUCTION

It is not uncommon for elderly individuals to have numerous houseplants and, possibly, to use anticholinesterase pesticides on them. Because of the confined space into which the pesticide may be sprayed, these individuals may be at great risk for toxic exposure. However, we are not aware of any published studies reporting such phenomena. The comparative study by Miller and Mitzel (1995) on individuals developing multiple chemical sensitivity after exposure to pesticides or to sick buildings indicated similar age distributions for the two groups. At present it seems, therefore, that there is not very much direct information on the exposure of aged individuals to OPs, so the question of whether aged individuals should be considered a susceptible population cannot be directly addressed. There is, however, a considerable amount of indirect information about cholinergic function and cognitive performance in aged individuals/animals. As will be seen in the pages that follow, this information can be used to develop specific hypotheses about the degree of susceptibility of aged populations; such hypotheses may be useful in directing further research on this important topic.

## CHOLINERGIC INDICES

Efficient cholinergic neurotransmission is dependent upon three key indices: the synthesizing enzyme, choline acetyltransferase (ChAT), the metabolizing enzyme acetylcholinesterase (AChE), and muscarinic cholinergic receptors (mAChR). ChAT is unique to cholinergic neurons and is responsible for converting choline + acetyl CoenzymeA into acetylcholine (ACh). Although ChAT is not a rate-limiting enzyme, with new ACh synthesis dependent more upon choline uptake (e.g., Russell and Overstreet, 1987), impairment of cholinergic transmission can occur if the enzyme activity is substantially lowered. Changes in ChAT activity are frequently regarded as indices of the integrity of cholinergic neurons or

terminals rather than of the capacity of the enzyme to synthesize ACh. Therefore, when severe deficiencies were reported in individuals with Alzheimer's disease (e.g., Perry et al., 1978; Bartus et al., 1982), it was interpreted as a loss of cholinergic neurons (Drachman and Leavitt, 1974; Bartus et al., 1982). Since cholinergic transmission may be compromised in such individuals with a dramatic loss of ChAT, it would be predicted that they might benefit from treatment with drugs, which inhibit AChE activity, as cholinergic transmission would be normalized. Indeed, the use of AChE inhibitors as potential therapeutic agents in the treatment of Alzheimer's disease has a long history and the recent success of one of these agents, metrifonate, will be briefly reviewed in a later section. Theoretically, individuals with severely compromised cholinergic transmission as a consequence of the loss of ChAT activity would be less at risk for the effects of organophosphate (OP) anti-AChE agents; a similar, but less straightforward case might be made for the aged, whose decline in ChAT activity relative to mature adults is less dramatic. To date, we are not aware of any studies, which have directly compared the responses to OPs from young, mature, and aged humans. Nor have there been any such comparisons in animals, although some investigators have examined other cholinergic agents (Pedigo and Polk, 1985; Pedigo et al., 1984) and others have recently studied one OP in young and mature rats (Moser and Padilla, 1998).

Aged animals and humans also exhibit a reduction in AChE, the enzyme responsible for the breakdown of ACh into choline and acetic acid (Bartus et al., 1982). As with ChAT activity, the loss of AChE activity is more severe in Alzheimer's patients and AChE activity has been detected in neurofibrillary tangles, one of the anatomical hallmarks of Alzheimer's disease (Sunderland et al., 1995). This latter observation has led to the suggestion that neurofibrillary tangles represent, in part, degenerating cholinergic terminals. Theoretically, it would be

predicted that individuals or animals with lowered AChE activity would be more sensitive to the effects of OP anti-AChE agents. However, as indicated above, there does not appear to be any literature, which relates to this topic.

A final cholinergic index that is commonly assessed in both animals and humans is the concentration of mAChR. Although it is now recognized from messenger RNA studies that there are five subtypes of mAChRs, typically only the M1 and M2 subtypes have been studied biochemically by receptor binding approaches. Initially, studies were carried out with a nonselective ligand such as quinuclidinyl benzilate (QNB) and several reports of decreases in muscarinic receptors were described in a variety of brain regions (e.g. Pedigo et al., 1984). When more selective approaches were used, it was suggested that the loss of M2 receptors was much greater than the loss in M1 receptors in Alzheimer's disease (Mash et al., 1985). Because M2 receptors are frequently located presynaptically and M1 receptors are located postsynaptically, this observation was interpreted as confirmatory evidence for the loss of cholinergic neurons and the relatively intact nature of cholinoceptive neurons (e.g. Mash et al., 1985).

In order to predict the relative risk of aged individuals/animals to OPs, it is important to understand the detailed nature of these receptor alterations. If, for example, the receptor losses are confined to the presynaptic M2 receptors, then there could be less feedback inhibition of cholinergic transmission. Consequently, there would be a greater functional effect of the ACh built up due to inhibition of AChE by the OPs and the aged individual would be more sensitive to OPs. If, on the other hand, there were also a loss of postsynaptic mAChRs (M1 or M2), the built up ACh would have a reduced functional effect and the individual would be less sensitive to OPs.

Because there are changes in all three of these important cholinergic indices during aging as well as in Alzheimer's disease, it is difficult to predict the relative risk of a particular individual. The risk would be, in part, dependent upon the cholinergic balance achieved after all of the changes are summed. Table 1 summarizes the commonly reported changes in these cholinergic indices and the likely risk to OPs based on several hypothesized types of changes. It may well be that there will be individuals at varying types of cholinergic balance in the aged population, so the most solid prediction that might be made about their responses to OPs is that the variability would be much greater than that seen for young or mature adults.

## CHOLINERGIC SENSITIVITY

It is very common for aged individuals to be more sensitive to many drugs. A commonly reported example is their inability to tolerate the side effects of tricyclic antidepressants (Salzman et al., 1995). In particular, the anticholinergic side effects such as dry mouth and blurred vision may become intolerable. It is possible that this increased sensitivity might be related to the observation of reduced mAChRs reported above (e.g., Pedigo et al., 1984; Mash et al., 1985). However, there could also be pharmacokinetic mechanisms related to metabolism (von Moltke et al., 1995; Salzman et al., 1995). Pedigo and colleagues have explored this issue by studying the hypothermic effects of the selective muscarinic agonist, oxotremorine, in young, (3 mo), mature (9 mo) and aged (27 mo) rats. Temperature is mediated by M2 receptors in the hypothalamus, so they also estimated the number of hypothalamic M2 receptors by QNB binding. The basic findings were paradoxical: Even though the aged rats had fewer mAChR receptors, they exhibited a greater hypothermic response to oxotremorine. Such a finding of a mismatch between receptor number and response to the relevant receptor agonist is becoming

increasingly common (e.g., Bushnell et al., 1993; Daws and Overstreet, 1999; Knapp et al., 1998) and suggests that predictions solely on the basis of receptor differences may not be correct.

Another example of this problematic issue of receptor levels and drug response is illustrated by some collaborative work carried out with Roger Russell in Don Jenden's laboratory at UCLA. Jenden and Russell had been studying the behavioral and biochemical effects of alkylating analogs of oxotremorine. These compounds acutely interact with the mAChR to produce agonist-like responses but then alkylate the receptor, thereby reducing the functional levels for several days (Russell et al., 1986a,b). Because the mAChR concentrations were still reduced by as much as 50% four days after the acute treatment, it was predicted that rats treated with the alkylating analog (BM123) would be less sensitive to soman, an OP nerve gas, because muscarinic blockers such as atropine have some protective effects. However, the rats treated with the alkylating analog of oxotremorine were more sensitive to soman, as indexed by a greater decrease in core body temperature (Jenden et al., 1985). Consistent with their greater sensitivity to soman, the BM123-treated rats had a greater suppression of brain AChE activity, even though BM123 had no effect on AChE activity itself (Jenden et al., 1985). Thus, the alkylating analog of oxotremorine had more complex effects than could be predicted by its simple alkylation of the mAChR. The exact mechanisms underlying this synergism between BM123 and soman have yet to be elucidated.

Moser and Padilla (1998) have recently completed a comprehensive behavioral and biochemical comparison of young (17-day old) and adult (70-day old) rats treated acutely with the OP, chlorpyrifos. The young animals were more sensitive to the behavioral effects, but also exhibited greater adaptations, as indicated by a greater down-regulation of mAChR and a faster recovery of AChE. Thus, young rats appear to be at greater risk for adverse effects from

exposure to OPs. However, as indicated above, there have been essentially no studies on the effects of OPs in aged rats. The techniques and procedures developed by Moser and Padilla (1998) could be usefully applied to this population.

Overall, therefore, one might predict that aged individuals would be more sensitive to the effects of OPs because they are more sensitive to the effects of a variety of other drugs and aged rats are more sensitive to oxotremorine. The reported decrease in mAChR with aging does not appear to have a functional consequence of reduced sensitivity to muscarinic agonists or AChE inhibitors.

## CHOLINERGIC ADAPTATIONS DURING CHRONIC OP TREATMENT

There is an extensive literature on the adaptation of young and mature animals to OPs which have been administered chronically and several reviews are available (e.g., Costa et al., 1982; Russell and Overstreet, 1987). In a typical experiment, AChE activity is maintained at a constant low level and the behavioral response is affected initially but recovers to baseline (e.g., Russell et al., 1971a,b). However, the rate of tolerance development appears to be more rapid for relatively simple responses but prolonged for more complex behavioral responses (Russell et al., 1971c). In fact, the ability of the rat to perform a complex alternation task never did recover to baseline during chronic DFP treatment (Overstreet et al., 1974). In other studies investigators were able to lower AChE activity at a slow enough rate so that no overt behavioral consequences were observed (Chippendale et al., 1992; Overstreet, 1974); yet these animals were confirmed to have become tolerant because of their altered responses to muscarinic agonists and antagonists.

Indeed, it has been commonly observed that animals chronically treated with OP anti-ChEs exhibit reduced sensitivity to muscarinic agonists and increased sensitivity to muscarinic antagonists (see Russell and Overstreet, 1987, for review). Schiller (1979), working in our

laboratory, was one of the first to show that these altered drug sensitivities might relate to a down-regulation of mAChRs which occurs during chronic treatment with OPs. This finding has been replicated on numerous occasions (see Russell and Overstreet, 1987), but several investigators have questioned whether a simple decrease in mAChRs can completely account for the development of tolerance to these agents (see Bushnell et al. 1993; Smolen et al., 1986). Thus, as indicated above, questions have been raised about the ability of the number of mAChRs to predict an animal's response to cholinergic agonists and/or anti-AChEs.

Because some more complex behaviors do not appear to adapt during chronic OP administration and because humans apparently exposed to relatively low levels of anti-AChEs may complain of memory problems (Miller and Mitzel, 1995), some investigators have explored the consequences of chronic low level exposure of OPs on complex cognitive behavior. Bushnell et al. (1991) used a complex matching-to-sample, food-motivated task to demonstrate that only the matching behavior was compromised during chronic treatment with DFP. Interestingly, the behavior did not begin to deteriorate until there was evidence for a subsensitive hypothermic response to oxotremorine and a decrease in mAChR. These findings are, therefore, reminiscent of the early work with higher doses of OPs where it appeared that the mAChR down-regulation offered a simple, perhaps complete explanation of the observation. The only difference in these results is that the mAChR decrease is now maladaptive in that the complex cognitive behavior is compromised. More recently, Bushnell et al. (1993) reported on the effects of Chlorpyrifos (CPF), an OP pesticide, which is converted slowly to an oxon, the active AChE inhibitor. They also observed reductions in the ability of the rats to perform the matching response which were associated with changes in AChE activity and mAChRs. However, the behavior recovered before the biochemical measures returned to normal. Furthermore, although

there were substantial decreases in mAChR in the hippocampus, they could not detect any differences in the cognitive effects of cholinergic agonists and antagonists (Bushnell et al., 1994). Thus, another mismatch between mAChR and behavioral response has been reported.

Whether or not aged individuals or animals would be more likely to experience cognitive deficits during chronic low level exposure to OPs has not yet been addressed, as far as we know. However, Pedigo (1988, 1994) has conducted an experiment with other cholinergic agents which bears on this issue. Adult (3 mo), mature (9 mo), and aged (27 mo) rats were chronically infused icv with low levels of artificial CSF, oxotremorine, a muscarinic agonist, or methyl atropine, a muscarinic antagonist. The adult rats exhibited the expected up-regulation following chronic exposure to methyl atropine and down-regulation following exposure to oxotremorine. There were parallel increases and decreases in the hypothermic responses to oxotremorine, so there was a correlation between receptor change and functional response. In contrast, the aged rats did not exhibit either a change in QNB binding or a change in the hypothermic response to oxotremorine. It appears, therefore, that the aged brain is less plastic than the young brain. This study also confirmed the earlier reported mismatch, with the aged rats treated by artificial CSF exhibiting a larger response to oxotremorine but having reduced mAChRs. If the aged human exhibits a similar degree of reduced plasticity, one would predict that they might exhibit a continued supersensitive response to OPs.

## METRIFONATE – AN OP TREATMENT FOR ALZHEIMER'S DISEASE?

Given the literature indicating that aged rats are more sensitive to cholinergic agonists (Pedigo et al., 1984), it may seem surprising that so much effort has been invested in developing anti-AChEs for the treatment of Alzheimer's disease. However, it must be remembered that the binding studies suggested that the M1 mAChR subtype may be relatively intact (Mash et al.

1985) and there are no appropriate directly acting receptor agonists available. There are some data suggesting that the M1 receptor link to its second messenger, phosphatidyl inositol, is impaired in aged animals (Crews et al., 1994). The gradual deterioration of cognitive behavior and the severity of the condition make Alzheimer's disease a fruitful area for the development of therapeutic agents having long-term benefits. Over the years a variety of agents have been used, including tacrine, huperzine, heptylphysostigmine, and metrifonate (Jann, 1998). There has been an increasing rich supply of recent research articles on metrifonate, an OP, so the rest of this section will focus on this compound.

Metrifonate is an OP that is converted to an active metabolite, 2.2-dimethyl dichlorovinylphosphate (DDVP), which binds to AChE irreversibly (Holmstedt et al., 1979). Its use as a treatment for Alzheimer's patients was stimulated by the observation that it has been used safely in the treatment for parasites for over 30 years (Cerf et al., 1962). An initial open trial in 20 Alzheimer's patients by Becker et al. (1990) reported positive results and has led to more substantial studies of the effects of this agent on memory and cognitive function in both animal models and in Alzheimer's patients.

Jann (1998) has recently reviewed the preclinical effects of metrifonate on a variety of animal models of memory dysfunction. Regardless of the manner by which the memory is disrupted, in either aging animals, scopolamine-injected animals, or animals with lesions, metrifonate has led to a cognitive improvement. This has occurred even though comparative anti-AChE inhibitors such as tacrine may have been ineffective.. Thus, there has accumulated a solid preclinical database that supports the therapeutic potential of metrifonate as an agent to improve cognitive function in Alzheimer's patients.

Recently the results of two large-scale, placebo-controlled, multicenter trials of the safety and efficacy of metrifonate in treating Alzheimer's patients have been reported (Cummings et al., 1998; Morris et al., 1998). Each study involved the examination of over 400 patients with mild to moderate Alzheimer's disease during long-term (10-24 weeks) treatment with placebo or varying doses of metrifonate. In both studies, using an intent-to-treat design, the metrifonate-treated patients performed significantly better on the Cognitive Subscale of the Alzheimer's Disease Assessment Scale. There were also improvements in global function. There was no evidence of liver toxicity and the predominant side effect, gastrointestinal disturbance, was relatively infrequent and of a mild nature (Cummings et al., 1998; Morris et al., 1998). There were several differences between the two studies, which should be highlighted, however. Cummings et al., (1998) conducted a dose-effect study over a shorter treatment period than did Morris et al. (1998). They reported that the highest dose of metrifonate used (0.65 mg/kg) significantly improved cognitive function, while those maintained on placebo tended to deteriorate (Cummings et al., 1998). The groups receiving the intermediate doses (0.2 and 0.3 mg/kg) had intermediate scores for cognitive function. Morris et al. (1998) conducted trials over a longer period with the 0.65 mg/kg dose and also reported significant differences in cognitive function between metrifonate- and placebo-treated patients. However, a closer examination of these data reveals that these findings seem to be largely the consequence of deterioration in performance of the placebo treated patients. The metrifonate-treated patients had rather stable but very small improvements in performance (Morris et al., 1998). Thus, it might be more appropriate to interpret this finding as evidence for metrifonate preventing the deterioration in cognitive performance that accompanies Alzheimer's Disease. Regardless of the interpretation, it is clear that metrifonate has a substantial therapeutic benefit.

All of the published reports suggest that metrifonate is a well-tolerated and safe anti-AChE agent which can be used not only for the treatment of tropical parasites but also for the treatment of Alzheimer's Disease. There has not been any information from these reports which suggests that aged individuals, including Alzheimer's patients, exhibit greater responses to metrifonate.

## AN ANIMAL MODEL OF CHOLINERGIC HYPOFUNCTION

Study of aged animals can take a very long time and/or be very expensive because of the high costs in obtaining and/or maintaining such animals. Therefore, the availability of an animal model of the cholinergic hypofunction typically seen in aged animals/humans could provide an opportunity to explore several critical hypotheses not yet addressed by other investigators. Roger Russell and his colleagues set about developing such a model in the late 1980's at UCLA. By feeding rats a diet reach in N-aminodeanol (NaDe), which competes with choline for ChAT and results in a false transmitter which is much less efficient than ACh, Russell et al. (1990) were able to produce rats with dramatic decreases in ACh levels. Interestingly, these animals also exhibited decreases in ChAT and mAChR and therefore resemble the aged rats and humans. The rats maintained on NaDe also exhibited a variety of behavioral deficits, including passive avoidance memory, so once again the animal model mimicked the observation seen in humans. In a subsequent study, it was found that a reversal of the diet led to a recovery of the biochemical measures within 30 days, but the behavioral measures had not recovered (Russell et al., 1992). Therefore, there must have been changes in the NaDe-exposed rats which are unrelated to mAChRs and recent studies have explored several hypotheses (Russell, 1996).

This model system, mimicking as it does both the behavioral and biochemical findings in aged animals, would appear to be an ideal model to use to explore the issues of whether aged

animals/humans are at greater risk to OPs. As far as we know, these rats have not been used to explore this issue as yet. Instead, they have been used to investigate the mechanisms underlying the long-term behavioral deficits (Russell, 1996) and/or possible treatments to overcome these deficits (Russell et al., unpublished manuscripts). Since there are decreases in both ChAT activity and mAChR concentrations, one would predict that rats exposed to NaDe would be less sensitive to OPs. But it must be remembered that rats treated with alkylating analogs of oxotremorine and having reduced mAChRs were more sensitive to soman (Jenden et al., 1985) . The experiments must be done before any firm conclusions can be made.

## CONCLUSIONS

Faced with this complexity of literature from several very different areas, one is reminded of a favorite Australian expression. The conclusion to this paper might very well be "I haven't got a clue, mate." However, Australians are typically argumentative and for every one who utters the phrase above there will be another who says "Hangonaminit (Hang on a minute)". Let's look at the evidence in a more systematic way. Table 2 itemizes the findings favoring an increased sensitivity of the aged to OPs on at the top and those favoring a decreased sensitivity at the bottom. As indicated above, in some cases we need to consider not only the biochemical index but also the anatomical location of the index (pre vs. postsynaptic). There is evidence predicting either reduced or increased sensitivity of aged individuals to OPs, so there is a clear need for further study. The four-phase research program recommended by Russell (1971) in his review paper on environmental quality and behavioral adjustment is still appropriate today. Two specific experiments would be of particular importance. The methods of Moser and Padilla (1998) could be adapted to compare the responses of mature and aged rats to chlorpyrifos, for example. The methods and procedures of Bushnell et al. (1991,1993,1994) could be used to

study cognitive and other behaviors of mature and aged rats during chronic treatment with OPs. If aged rats do indeed have reduced receptor adaptation (Pedigo, 1988), then these rats may develop tolerance to the hypothermic and depressing effects of OPs less readily (Russell and Overstreet, 1987), and may not exhibit the cognitive deficits seen with chronic exposure in younger animals (Bushnell et al., 1991,1993). Although these experiments could be carried out in aged animals and their controls, it might be very revealing to test the effects of OPs in rats exposed to control or NaDe diets. In the mean time, it is probably wise to err on the conservative side and view the aged population as having an increased risk to the effects of OPs.

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Table 1

Changes in Cholinergic Indices with Aging and Predicted Responses to OPs

A. Predictions from Changes in Single Index

| Index | Change with Aging | Predicted OP Response |
|---|---|---|
| ChAT | Decrease | Decrease |
| AChE | Decrease | Increase |
| mAChR | Decrease | Increase/Decrease[1] |

B. Predictions from Changes in Multiple Indices

| Relative Changes in Indices with Aging | Predicted OP Response |
|---|---|
| ChAT decrease > AChE decrease | Decrease |
| AChE decrease > ChAT decrease | Increase |
| Pre-mAChR decrease> Post-mAChR decrease | Increase |
| Post-mAChR + ChAT decrease > Pre-mAChR + AChE decrease | Decrease |

[1]Direction of response depends on relative changes in pre-and post-synaptic mAChRs

Table 2

Summary of Evidence Suggesting Increased or Decreased Responses to OPs in the Aged

A. Data Suggesting Increased Risk of Aged to OPs

1. Decrease in AChE with aging
2. Decrease in mAChR (M2) with aging
3. Increase in sensitivity to muscarinic agonists with aging
4. Increase in sensitivity to many other drugs with aging

B. Data Suggesting Reduced Risk of Aged to OPs

1. Decrease in ChAT with aging
2. Decrease in mAChR (M1)/PI coupling with aging
3. Decreased receptor adaptation with aging
4. Therapeutic benefit of anti-AChEs in Alzheimer's Disease

# SUPERSENSITIVE GROWTH HORMONE RESPONSES TO PYRIDOSTIGMINE IN A GENETIC ANIMAL MODEL OF DEPRESSION WITH CHOLINERGIC HYPERSENSITIVITY

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Abstract

Exaggerated growth hormone responses to pyridostigmine have been observed in humans with a variety of disorders, including depression. The present study examined growth hormone responses to pyridostigmine in a genetic animal of depression, the Flinders Sensitive Line (FSL) rat, which has been selectively bred for increased sensitivity to cholinergic agonists and anticholinesterases. Challenges with the centrally acting cholinergic agonist, oxotremorine, confirmed that the FSL rats exhibited an exaggerated hypothermic response compared to the Flinders Resistant Line (FRFL) rats; randomly bred Sprague-Dawley (SD) rats showed intermediate hypothermic responses to oxotremorine. Compared to FRL rats, both FSL and SD rats exhibited increased growth hormone responses to pyridostigmine at low and intermediate doses. In contrast, none of the three groups of rats exhibited a hypothermic response to pyridostigmine, confirming that this anticholinesterase agent does not penetrate the blood-brain barrier. These findings suggest that the receptor sites responsible for the effects of pyridostigmine on growth hormone probably lie outside of the blood-brain barrier. The exaggerated growth hormone response in the FSL rat supports this rat as a genetic animal model of depression.

Introduction

Pyridostigmine is a peripherally acting anticholinesterase agent that has been used for a variety of purposes in biomedical sciences. In the past, it has been widely used as a treatment for myasthenia gravis (e.g., Keesey, 1998). More recently, it has been used as a tool to investigate abnormalities in growth hormone regulation in a variety of human disease states, such as depressive disorders, schizophrenia, and Alzheimer's Disease (Chaudhuri et al., 1997; Cooney et al., 1997a,b; Ghigo et al., 1993; Lucey et al., 1993; O'Keane et al., 1992, 1994). Pyridostigmine is also one of the prophylactic medications that was taken by individuals participating in the Persian Gulf War (Jamal, 1998; Keeler et al., 1991; Shen, 1998). Because of the large individual differences in growth hormone responses to pryridostigmine in humans, it is possible that a single dose regimen may not be equally protective against nerve gas exposure. The purpose of the present study was to examine the dose-dependent effects of pyridostigmine in groups of rats known to differ in cholinergic sensitivity in order to select a dose that could be used in a prophylactic study.

The Flinders Lines of rats have been developed from Sprague-Dawley rats by selective breeding for responses to the anticholinesterase agent diisopropyl fluorophosphate (DFP) and have been maintained by determining their hypothermic responses to the centrally acting cholinergic agonist, oxotremorine (Overstreet, 1993; Overstreet et al., 1995). The Flinders Sensitive Line (FSL) rats exhibit greater hypothermic responses to DFP and to cholinergic agonists than the Flinders Resistant Line (FRL) rats. These responses can be seen as early as temperature can be reliably measured (Daws and Overstreet, 1999), so they are probably innate. The FSL rats also exhibit elevated muscarinic receptors in hypothalamic, hippocampal and striatal regions of the brain (Daws and Overstreet, 1999; Overstreet et al., 1995). The increased cholinergic sensitivity of the FSL rats is reminiscent of the increased sensitivity of depressed individuals to cholinergic agonists (e.g.

Janowsky et al., 1994). However, the FSL rats also resemble depressed humans in having increased REM sleep; reduced locomotor activity; and exaggerated behavioral responses to stress (See Overstreet, 1993; Overstreet et al.,, 1995, for review). The fact that the exaggerated immobility of the FSL rats in the forced swim test can be counteracted by tricyclic antidepressants and selective serotonin reuptake inhibitiors (Overstreet et al., 1995; Caberlotto et al., 1998; Zangen et al., 1997, 1999) further support the FSL rat as a genetic animal model of depression. This rat model with increased cholinergic sensitivity provides an excellent opportunity to investigate the acute effects of pyridostigmine on growth hormone and its chronic prophylactic effects against organophosphate agents. The present study reports on the acute effects of pyridostigmine.

Because depressed individuals exhibit exaggerated growth hormone responses to pyridostigmine ( Cooney et al., 1997b; O'Keane et al., 1992), it was predicted that the FSL rats would be more sensitive to the effects of pyridostigmine than the FRL rats. To provide a broader understanding of the effects of pyridostigmine in the two selected lines, a group of commercially bred Sprague-Dawley (SD) rats were included for comparison and core body temperature and home cage activity were telemetrically monitored. The findings confirmed that pyridostigmine significantly altered only the growth hormone dependent measure and that the FSL rats had elevated responses compared to the FRL rats. Preliminary reports of these findings have been published elsewhere (Overstreet et al., 1997, 1998)

## Methods

### Animals

The FSL and FRL rats were selected from breeding colonies maintained in the Skipper Bowles Center for Alcohol Studies. The commercially bred Sprague-Dawley (SD) rats were purchased from Harlan (Indianapolis, IN) at 65 days of age. The rats were housed in groups or 3-5

in polycarbonate cages under standard housing conditions (22 oC, 50 % humidity) and a reversed light:dark cycle (lights off from 1000-2200).

Surgery

Recording of locomotor activity and core body temperature in freely moving rats was accomplished by the implantation of a transmitter weighing 7.0 g (Model TA-11ETA-F40-L20). This transmitter had temperature- and motion-sensitive elements and when actuated by passing a magnet along the rat's abdomen, transmitted information to a computer where it was stored using Data Quest IV software (Data Sciences, Inc., St. Paul, MN).

At about 70 days of age the rats were injected i.p. with sodium pentobarbital (35 mg/kg) to induce anesthesia for implanting the telemetry transmitters, which provided continuous monitoring of core body temperature and general activity. The fur over the ventral abdominal area was clipped and a 3-cm longitudinal incision was made along the midline about 1 cm below the sternum. The radiotransmitter was inserted into the abdominal cavity and sutured to the peritoneal wall with 4-0 silk thread. After testing the transmitter with an AM receiver, the skin was closed (Rezvani et al., 1994). The rats were placed in single polypropylene cages after surgery and were closely monitored until they were active.

Procedure

After a one week period to allow full recovery (Rezvani et al., 1994), the FSL, FRL and SD rats were adapted to the home cages for at least 24 hr and then injected s.c. with a mixture of peripherally acting methyl atropine (MA, 2.0 mg/kg) and oxotremorine (OXO, 0.2 mg/kg) to determine hypothermic responses. This treatment was given to insure that each group of rats were either sensitive (FSL) or resistant (FRL) to a well characterized cholinergic agonist. This information is necessary to interpret the hypothermic responses to pyridostigmine.

Approximately three days after the MA/OXO challenge, the rats were given pyridostigmine (PYR) bromide by gavage (3 ml/kg). The design called for four groups (vehicle and 4, 12, 36 mg/kg), with ten rats per group. The animals were run in squads of 10 rats, the capacity of the computer, in a counterbalanced order. The average temperatures and general activity counts recorded during the hour preceding the gavage and those recorded at approximately 30 min after the treatment were used in statistical analyses.

The rats were sacrificed by decapitation exactly 30 min after the oral administration of pyridostigmine, any signs of diarrhea were noted, and blood was collected into centrifuge tubes. The tubes were centrifuged and the plasma was collected and stored at -20 °C for later determination of growth hormone levels.

Growth Hormone Assay

The concentrations of growth hormone were estimated by using a kit provided by NIDDK. Preliminary tests confirmed that this radioimmunoassay was linear over the range of values expected to be obtained in these rats.

Statistical Analysis

The data were initially analyzed by 3-factor ANOVAs, with gender, line, and dose of pyridostigmine as the three main factors. When there were no significant effects for one of the main factors, such as gender, then the data were reanalyzed using two-way or one-way ANOVAs. When ANOVAs were significant, follow-up Tukey's tests were conducted.

## Results

The outcome of the challenge with the centrally acting muscarinic agonist, oxotremorine, is illustrated in Figure 1. There were highly significant gender ($F[1,187] = 83.71$, $p < 0.0001$) and line

($F[2,187] = 149.3$, $p < 0.001$) effects. Within each line, the female rats exhibited greater decreases in temperature than their male counterparts (See Figure 1). The FSL rats exhibited dramatic decreases in core body temperature, and there was essentially no overlap in their distribution with that of the FRL rats, which exhibited very small decreases in temperature (See Figure 1). The response of the SD rats was intermediate. Overall, it is clear that both the FSL and FRL rats differed from the SD rats. Thus, the FSL and FRL rats are truly sensitive and resistant, respectively, to the hypothermic effects of oxotremorine relative to this SD group.

The effects of pyridostigmine on growth hormone, the key dependent measure, are illustrated in Figure 2. There were no significant gender differences, so the data were combined to provide larger sample sizes. Analysis of these data by two-way ANOVA confirmed that there were significant dose ($F[3,235] = 15.95$, $p < 0.001$) and line ($F[2,235] = 6.34$, $p < 0.01$) effects. All groups exhibited an inverted U-shaped function, with the intermediate dose inducing the greatest increase in serum growth hormone. However, the degree of elevation was significantly higher ($p < 0.05$) in the FSL rats compared to the FRL rats. Interestingly, the SD rats resembled more closely the FSL rats than the FRL rats.

There were few significant gender or line effects of pyridostigmine treatment on temperature or home cage activity, two measures that are known to be influenced by centrally acting cholinergic agonists. As indicated in Table 1, the females exhibited a greatest degree of hyperthermia after 4 mg/kg pyridostigmine. All groups exhibited hyperthermia after isotonic saline, but not after 36 mg/kg pyridostigmine; there were no remarkable line or gender effects.

## Discussion

The oxotremorine challenge confirmed the large differences in cholinergic sensitivity between the FSL and FRL rats. This finding indicates that the innate differences in cholinergic sensitivity have been maintained even though the selection pressure has not been continued. In any case, the present results are entirely consistent with the many frequent reports of exaggerated cholinergic sensitivity in the FSL rat (Overstreet, 1993; Overstreet et al., 1995). The present findings also clearly indicate that the FRL rats are different from the SD rats, suggesting that both resistant and sensitive lines of Flinders rats have been established. Such a conclusion was not possible in many of the previous experiments because only the FSL and FRL rats were used. Early in the selection process, when SD rats were included, the FRL rats did not differ from the SD rats (e.g., Russell et al., 1982). Whether or not the commercially bred SD rats resemble the FSL or the FRL rats or are intermediate may depend upon the supplier of SD rats. For example, we have recently shown that the FSL rat is more sensitive to methacholine challenges in an experimental asthma protocol than either the SD or FRL rats, which do not differ from each other (Djuric et al., 1998). Other studies have directly compared SD rats from different suppliers and have noted large differences (Balcells-Olivero et al., 1997, 1998; Trujillo et al., 1998). These varying responses in different groups of SD rats were not known at the time these studies were planned. Their existence suggests that we should temper somewhat our conclusion that rat lines both resistant and sensitive to cholinergic agonists have been developed.

Compared to the dramatic decreases in temperatures seen with oxotremorine in the FSL and SD rats, those seen after pyridostigmine were minuscule. Because central stimulation of cholinergic mechanisms leads to hypothermia, pyridostigmine is unlikely to have central actions in these rats because its predominant effect is hyperthermia. Such an outcome is consistent with what we know

about the pharmacology of these two agents. Oxotremorine is a tertiary amine and easily penetrates the CNS; in contrast, pyridostigmine is a quaternary anticholinesterase and has difficulty entering the CNS. The small hyperthermia induced by lower doses of pyridostigmine is most likely due to as yet unidentified peripheral mechanisms.

The fact that there was not even a hint of an hypothermic response in the FSL rats after challenge with pyridostigmine suggests that the blood-brain barrier was intact in these rats. Because the FSL rat is a genetic animal model of depression which is more sensitive to behavioral stressors and because stress has been reported to weaken the blood-brain barrier (e.g., Friedman et al.,, 1998), it was considered possible that pyridostigmine might have a hypothermic effect in the FSL rats. However, the data clearly do not support such a view.

The exaggerated growth hormone response to pyridostigmine in the FSL rats is entirely consistent with all of the previous literature indicating cholinergic hypersensitivity in these rats compared to the FRL rats (Overstreet, 1993; Overstreet et al., 1995). It is unlikely that this difference could be related to differences in the integrity of the blood-brain barrier because, as indicated above, there were no hypothermic effects of pyridostigmine in any group of rats. Rather, they are more likely due to differences in muscarinic receptor function between the FSL and FRL rats. It is not exactly clear where these muscarinic receptors are located, but our findings suggest that they are located outside of the blood-brain barrier because of the lack of hypothermia induced by pyridostigmine. Other recent studies have shown that the exaggerated cholinergic sensitivity exhibited by the FSL rats can be observed for peripheral responses in the smooth muscles of the gut and the airways (Djuric et al., 1995, 1998). When these findings are combined with the present results, it must be concluded that the cholinergic hypersensitivity of the FSL rats is pervasive.

Although there are clear-cut differences in growth hormone responses to pyridostigmine in the FSL and FRL rats, the SD rats exhibited responses more similar to the those of the FSL rats. When all three groups are considered, it would be concluded that the FRL rats are more resistant but that the FSL rats are not more sensitive than the SD rats. However, as indicated above, this outcome/conclusion may be a product of the supplier chosen for the SD rats. There are differences in responses to various drugs between Harlan SD, Holtzman SD, and Charles-River SD (Balcells-Olivero, 1997, 1998; Trujillo et al., 1998), but no one has studied these substrains after cholinergic agents. Regardless of how the reference group responded, it is clear that there is a substantial difference in growth hormone response to pyridostigmine between the FSL and FRL rats.

The mechanisms underlying the cholinergic regulation of growth hormone have not been fully established. It has been proposed that cholinergic agonists stimulate growth hormone output by inhibiting somatostatin (Giustina et al., 1995). Some investigators have argued that differences in somatostatin may account for differences in growth hormone responses to pyridostigmine (Giustina et al., 1995). The present findings, by demonstrating an increased growth hormone response to pyridostigmine in the FSL rats with other exaggerated responses to cholinergic agonists and increased cholinergic receptors (Djuric et al., 1995; 1998; Overstreet et al., 1995), suggest that an abnormality in muscarinic receptor function may also participate in exaggerated growth hormone responses to pyridostigmine.

Although an altered growth hormone response to pyridostigmine has been detected in several human disease states (Chaudhuri et al., 1997; Cooney et al., 1997a,b; Ghigo et al., 1993; Lucey et al., 1993; O'Keane et al., 1992, 1994;), the increased response reported in depressive disorders (Cooney et al., 1997b) is particularly germane to the present study. Like the FSL rats, depressed individuals have been reported to exhibit supersensitive responses to cholinergic agonists and

anticholinesterase agents (Janowsky et al., 1994). Thus, both depressed individuals and FSL rats seem to have a pervasive cholinergic hypersensitivity. The fact that the increased cholinergic responsiveness can be observed in peripheral tissues as well as the CNS (Djuric et al., 1995, 1998) may account for why there is a high incidence of peripheral problems in depressed individuals, such as irritable bowel (Gruber et al., 1996). The FSL rats are also more sensitive to the effects of allergens (Djuric et al., 1995, 1998) and there has been some suggestion that a hypersensitive cholinergic system may help account for the link between allergies and depression (Marshall, 1993).

In conclusion, the FSL rat exhibited an exaggerated growth hormone response to pyridostigmine compared to the FRL rats. This finding is entirely consistent with previous reports of cholinergic hypersensitivity in the FSL rats (Djuric et al., 1995, 1998; Overstreet et al., 1995). These data also further support the FSL rat as a genetic animal model of depression, because an exaggerated growth hormone response to pyridostigmine has been associated with depressive disorders (Cooney et al., 1997b).

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Table 1

Change in Core Temperature (°C) after Oral Administration of

Saline or Pyridostigmine in FSL, FRL and SD Rats

| Line/Sex | Dose of Pyridostigmine (mg/kg) | | | |
|---|---|---|---|---|
| | 0.0 | 4.0 | 12.0 | 36.0 |
| SD-male | +0.4±0.1 | +0.6±0.1[b] | +0.2±0.1 | 0.0±0.2 |
| SD-female | +0.5±0.1 | +1.0±0.1[a] | +0.5±0.1 | -0.2±0.2 |
| FSL-male | +0.3±0.3 | +0.3±0.2[c] | +0.9±0.4 | -0.2±0.2 |
| FSL-female | +0.4±0.2 | +0.7±0.1[b] | +0.8±0.2 | +0.1±0.2 |
| FRL-male | +0.3±0.2 | +0.2±0.1[c] | +0.3±0.2 | +0.1±0.2 |
| FRL-female | +0.3±0.2 | +0.4±0.2[b,c] | +0.8±0.1 | +0.2±0.1 |
| One-Way ANOVA | 0.50 | 5.17** | 2.55 | 0.91 |

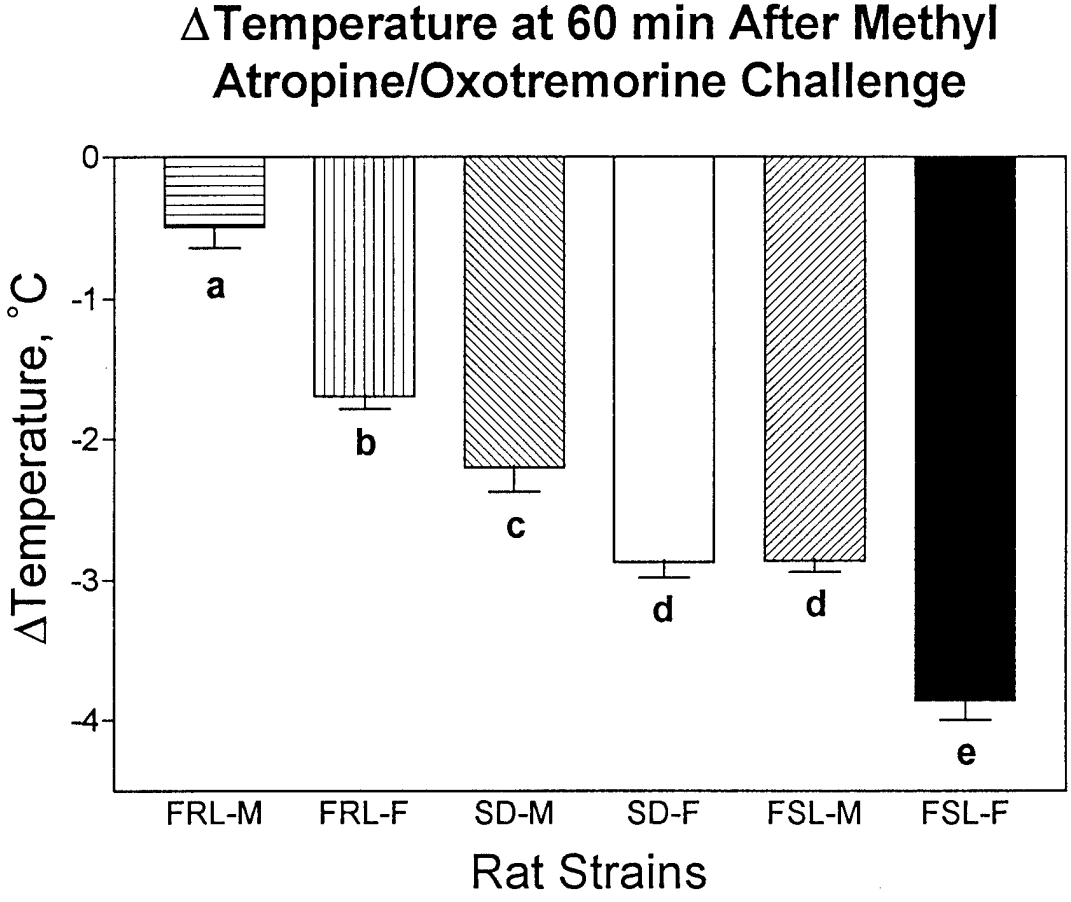
**Significant differences, $p < 0.01$

Groups with different letters are significantly different, $p < 0.05$, Tukey's test.

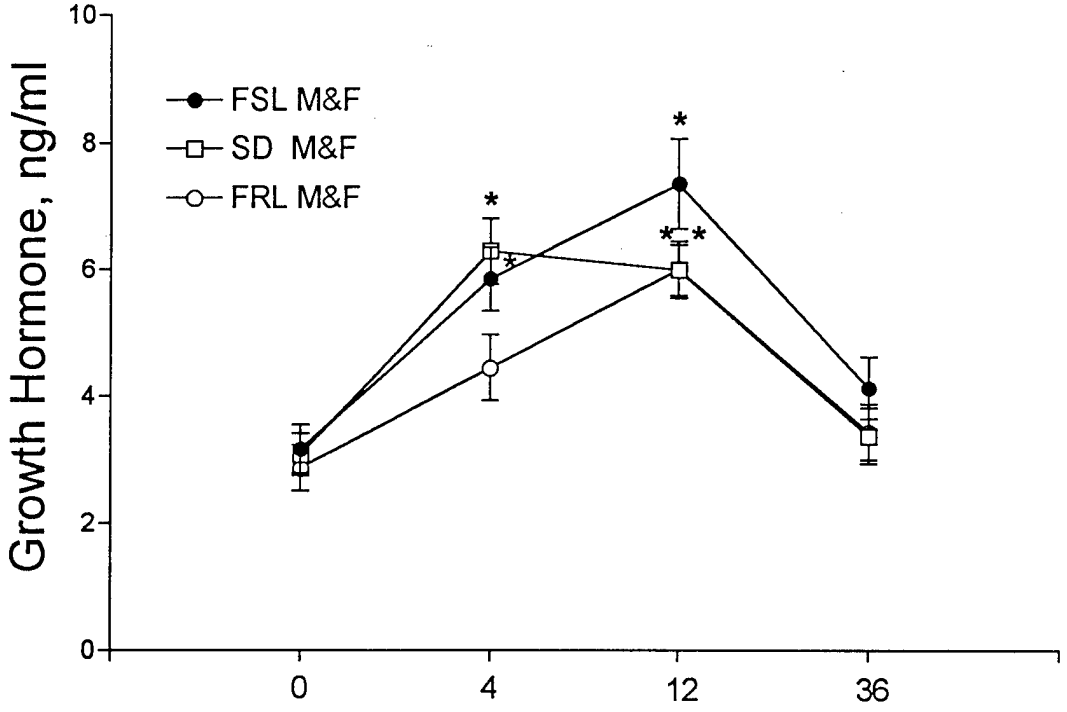
## Figure Captions

Figure 1. Strain and Gender-Dependent Effects of Oxotremorine in FSL, FRL and SD Rats. After the recording of baseline temperatures, rats were injected sc with a mixture of 2 mg/kg methyl atropine nitrate and 0.2 mg/kg oxotremorine sesquifumarate. The scores represent the mean decrease from baseline temperature (°C) for 25-30 rats at 60 min after the injection. Different letters indicate that the groups are significantly different from each other according to Tukey's tests. See text for additional statistical analyses.

Figure 2. Dose-Dependent Effects of Pyridostigmine on Serum Growth Hormone Levels in FSL, FRL and SD Rats. Rats were treated with pyridostigmine or saline by gavage 30 min prior to sacrifice by decapitation. Blood was collected in heparinized tubes and stored frozen at -20 °C until assayed by a kit from NIDDK. Values represent the mean values for 9-15 rats. *Significantly different from saline treatment. See text for additional statistical analyses,


ΔTemperature at 60 min After Methyl Atropine/Oxotremorine Challenge
ΔTemperature, °C
0
-1
-2
-3
-4
a
b
c
d
d
e
FRL-M
FRL-F
SD-M
SD-F
FSL-M
FSL-F
Rat Strains

## Effects of Pyridostigmine on Serum Growth Hormone in Flinders Rats




*Significantly different from 0 dose

# CHRONIC PRETREATMENT WITH PYRIDOSTIGMINE POTENTIATES THE HYPOTHERMIC EFFECTS OF ORGANOPHOSPHATE ANTICHOLINESTERASES

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Abstract

The present study sought to determine whether pyridostigmine would give equal protection to both genders of rats strains known to have innately different sensitivities to cholinergic agents and anticholinesterases. The male and female Flinders Sensitive Line (FSL), Sprague-Dawley (SD, obtained from Harlan), and Flinders Resistant Line (FRL) rats were chronically treated with saline or pyridostigmine (12 mg/ikg) by gavage for 14 days. Chjallenges with saline, chlorpyfos (CPF, 60 mg/kg, by gavage), or diisopropylfluorophosphate (DFP, 1 mg/kg, i.m.) were given 30 min afgter the last treatment. Temperatures were monitored telemetrically dduring the last three days of chronic treatment and for at least 2 hr after the challenges. As expected, the FSL rats exhibited greater decreases in temperature than the FRL rats after both challenge agents. The SD rats tended to exhibit intermediate hypothermic resonses. For most of the groups the decrease in body temperature after CPF or DFP occurred at a more rapid rate in the rats pretreated with pyridostigmine. When the changes in temperature one hr after the challenges were compared with those immediately before the challenges, all pyridostigmine-treated groups except for the FSL males challenged with CPF exhibited significantly greater changes than their saline-treated counteparts. By the end of 2 hr, these differences had largely disappeared. Thus, pyridostigmine did not protect any group of rats from the hypothermic effects of CPF or DFP. However, the incidence of diarrhea, which was mainly observed in the FSL and SD rats, was less in the pyridostigmine-pretreated groups. When rats were challenged with the directly acting muscarinic agonist after 10 days of chronic treatment, there were relatively few differences in the FRL and SD rats, but the FSL rats pretreated with pyridostigmine were more affected. Acute

pretreatment with physostigmine (4 mg/kg orally) was able to block the hypothermic effects of DFP. These findings indicate that pyridostigmine can protect animals against the peripheral effects of organophosphates but not their central effects.

Key Words: Pyridostigmine, Core Body Temperature, Chlorpyrifos, Diisopropylfluorophosphate, Cholinergic Hypersensitivity, Flinders Rats

## Introduction

Based on earlier reports that pyridostigmine had some protective effects against organophosphate nerve agents (Dirnhuber et al., 1979; French et al., 1979; Walday et al., 1993; Xia et al., 1981), many participants in the Persian Gulf War were given this peripherally acting anticholinesterase agent (Keeler et al., 1991). Quite a number of such participants have subsequently complained of a number of multi-organ symptoms that have been referred to as the Gulf War Syndrome and there is still considerable debate about the degree of contribution that pyridostigmine treatment has made to this Syndrome (Jamal, 1998; Shen, 1998). Because all individuals treated with pyridostigmine did not complain of symptoms, there most be factors other than mere exposure that are important. The current paper considers the possibility that genetic background might be important by examining the ability of pyridostigmine pretreatment to protect groups of rats with varying cholinergic sensitivity against organophosphate exposure.

The Flinders Line rats were developed from Sprague-Dawley rats by selectively breeding for hypothermic and other responses following administration of the organophosphate, diisopropyl fluorophosphate (DFP). The Flinders Sensitive Line (FSL) rat exhibits greater hypothermic responses to DFP as well as directly acting muscarinic receptor agonists and has a greater number of hippocampal and striatal muscarinic receptors than the Flinders Resistant Line (FRL) or Sprague-Dawley (SD) rats (Daws and Overstreet, 1999; Overstreet, 1993; Overstreet and Russell, 1982; Overstreet et al., 1979, 1984, 1995; Pepe et al., 1988; Rezvani et al., 1994). Because the FSL rat is more sensitive to the effects of a range of drugs, it has been proposed as a model of multiple chemical sensitivity (Overstreet et al., 1996), a condition which includes many symptoms

that overlap with those reported in individuals with the Gulf War Syndrome. Because of their exaggerated responses to cholinergic agonists, including pyridostigmine (Overstreet et al., 1997), it was predicted that the FSL rats might exhibit a smaller degree of protection from prophylactic pyridostigmiine treatment. To our surprise, all groups exhibited time-dependent greater hypothermic responses to organophosphates following pretreatment with pyridostigmine.

## Methods

### Animals

The FSL and FRL rats were selected from breeding colonies maintained in the Bowles Center for Alcohol Studies. The commercially bred Sprague-Dawley (SD) rats were purchased from Harlan (Indianapolis, IN) at 65 days of age. The rats were housed in groups or 3-5 in polycarbonate cages under standard housing conditions (22 °C, 50 % humidity) and a reversed light:dark cycle (lights off from 1000-2200) until surgery.

### Surgery

Recording of locomotor activity and core body temperature in freely moving rats was accomplished by the implantation of a transmitter weighing 7.0 g (Model TA-11ETA-F40-L20). This transmitter had temperature- and motion-sensitive elements and when actuated by passing a magnet along the rat's abdomen, transmitted information to a computer where it was stored using Data Quest IV software (Data Sciences, Inc., St. Paul, MN).

At about 70 days of age the rats were injected i.p. with sodium pentobarbital (35 mg/kg) to induce anesthesia for implanting the telemetry transmitters, which provided

continuous monitoring of core body temperature and general activity. The fur over the ventral abdominal area was clipped and a 3-cm longitudinal incision was made along the midline about 1 cm below the sternum. The radiotransmitter was inserted into the abdominal cavity and sutured to the peritoneal wall with 4-0 silk thread. After testing the transmitter with an AM receiver, the skin was closed. The rats were placed in single polypropylene cages after surgery and were closely monitored until they were active.

Procedure

An intermediate dose of 12 mg/kg pyridostigmine was selected as the chronic dose because it produced a significant elevation of growth in every group (Overstreet et al., 1997); it was gavaged in a volume of 3 ml/kg and the controls received an equivalent volume of isotonic saline orally for a total period of 14 days. Chronic treatment was initiated approximately 1-2 days prior to surgery to implant the transmitter. Surgery was performed in the afternoon, approximately 4-6 hr after the daily treatment with pyridostigmine and proceeded without incident. After a one week period of recovery, the rats were placed on the receivers for the monitoring of temperature and activity baselines for at least 48 hr prior to the challenge with the organophosphates. .

Exactly 30 min after the 14th treatment with pyridostigmine, the rats received one of three challenge treatments: Saline (3 ml/kg orally), chlorpyrifos (CPF; 60 mg/kg in 3 ml/kg orally), or DFP (1 mg/kg intramuscularly). These doses were selected to be intermediate in their hypothermic effects based on earlier literature (e.g., Nostrandt et al., , 1997; Overstreet et al., 1979). Corn oil and peanut oil were the vehicles for CPF and DFP, respectively. Temperatures were monitored for the next two hr and then the rats were sacrificed by decapitation. The blood was stored for the later determination of

cholinesterase activity and possible growth hormone levels and the brains were stored for the later determination of cholinesterase activity and muscarinic receptor binding. Only the temperature data will be communicated in this report.

The rats were also challenged with the directly acting muscarinic receptor agonist, oxotremorine (OXO), along with the peripherally acting antagonist, methyl atropine (MA). Initially, the challenge was conducted two days prior to the start of the chronic treatment phase, using a Physiotemp telethermometer and temperature probe. After the recording of baseline temperatures, the rats were given sc injections of the MA/OXO (2/0.2 mg/kg) mixture and core body temperatures were recorded at 30, 60 and 90 min after the injections. These recordings provided information about the sensitivity of the cholinergic system in the various groups prior to the start of chronic pyridostigmine or saline treatment.

When it became apparent that pyridostigmine was altering the hypothermic sensitivity to CPF and DFP, the timing of the MA/OXO challenge was changed to nine days after the initiation of the chronic treatment period (approximately one week after the implantation of the transmitters). Temperature was now monitored telemetrically and 48 hr of baseline and the complete time course of oxotremorine-induced hypothermia were recorded. This procedure was adopted so that the potential effects of chronic pyridostigmine on a cholinergic drug which is not dependent upon cholinesterase inhibition for its effects. Oxotremorine interacts directly with central cholinergic receptors (since its peripheral effects were blocked by MA) to induce its hypothermic effects.

Statistical Analysis

The data were analyzed by 2-factor ANOVAs, with gender/line (FSL, FRL, SD) and pretreatment (pyridostigmine, saline) as the two main factors. When ANOVAs were significant, follow-up Tukey's test were conducted.

## Results

Initial Cholinergic Sensitivity

The results of the MA/OXO challenges on core body temperature prior to the beginning of chronic treatment are summarized in Figure 1. Only the results for 60 min are illustrated because the pattern is virtually identical at 30 and 90 min. The FSL rats are clearly more sensitive to the hypothermic effects of MA/OXO and the FRL rats are resistant, both in reference to the FSL rats and the randomly bred SD rats. There were highly significant gender ($F[1,187] = 83.71$, $p < 0.0001$) and line ($F[2,187] = 149.3$, $p < 0.001$) effects. Within each line, the female rats exhibited greater decreases in temperature than their male counterparts (See Figure 1).

Chronic Pyridostigmine and Organophosphates

To illustrate the changes in temperature that occurred during the various treatments, the data have been presented as a series of graphs containing the last 4.5 hr prior to the last chronic treatment, the 30 min following this last chronic treatment and the 2 hr following the challenge treatment. Statistical analyses of the responses of the respective groups will be based upon data at specific time points, as described below.

The first set of figures illustrates that a saline challenge in the rats chronically treated with pyridostigmine or saline produced relatively few effects, as might be expected (Fig. 2A-F). However, there are distinct trends in the data for the some of rats chronically treated with pyridostigmine to have elevated or reduced temperatures relative to the saline-treated controls (See Fig. 2).

The second set of figures illustrates the hypothermic effects of CPF in rats chronically treated with pyridostigmine or saline. In five out of six of the groups the rats that had been chronically pretreated with pyridostigmine exhibited a more rapid decrease in temperature after CPF challenge (See Figure 3). As illustrated in Figure 4, DFP also produced a more rapid decrease in temperature in all of the rats chronically pretreated with pyridostigmine. These effects were quite striking. Thus, as indicated in Figures 3 and 4, almost all of the groups chronically pretreated with pyridostigmine exhibited more rapid decreases in temperature than the groups chronically pretreated with saline.

To evaluate these differences, the average temperatures of the various treatment groups at 1 and 2 hr after the acute challenge treatments of DFP and CPF (1.5 and 2.5 hr after the last pyridostigmine or saline treatment) were compiled in tabular form and analyzed by two-way Analysis of Variance, with line and pretreatment as the two main factors. These findings are summarized in Tables 1 & 2. Pretreatment effects were significant at 1 but not at 2 hr, while strain differences were significant at both time points. In all cases, the FRL rats were the most resistant, while the FSL rats were the most sensitive to DFP but equally as sensitive to CPF as the SD rats. These analyses confirm that the rats pretreated with pyridostigmine, regardless of line or sex, exhibited a more rapid decline in temperature than the rats pretreated with saline.

Chronic Pyridostigmine and Oxotremorine

Another approach to account for the differences in sensitivity in pyridostigmine- and saline-treated rats is to introduce a challenge to an agent whose effects are not dependent on cholinesterase inhibition. As indicated in Figure 5, it appears that chronic pyridostigmine treatment also sensitized some animals to the hypothermic effects of OXO, a directly acting cholinergic receptor agonist. Moreover, the pattern of temperature changes is different for OXO than for CPF or DFP. For all three drugs the peak change in temperature was fairly similar for rats pretreated with either pyridostigmine or saline. For OXO, there was a prolonged hypothermia in the pyridostigmine-treated FSL groups (Fig. 5B & 5E), while it was shorter in the SD males (Fig. 5D). In contrast, for CPF and DFP, there was a more rapid decline in body temperature in almost all groups (Fig. 3 & 4). Thus, the FSL rats, which are more sensitive to OXO already, are even more sensitive after being chronically treated with pyridostigmine.

Assessment of Diarrhea.

Diarrhea is a frequent symptom in animals exposed to anticholinesterase agents and is probably a reasonable index of peripheral cholinergic overstimulation. Evidence of diarrhea was observed at the time of sacrifice two hr after administration of the CPF, DFP or saline challenges and 2.5 hr after the last treatment with pyridostigmine or saline. There were no signs of diarrhea in the rats challenged with saline. CPF, which was orally administered at a intermediate dose of 60 mg/kg (Nostrandt et al., 1997), also produced relatively little diarrhea, with only two FSL male rats (one pretreated with pyridostigmine, one pretreated with saline) showing signs of diarrhea. The incidence of diarrhea was higher following the sc administration of 1 mg/kg DFP. Across all groups, a total of 2 out

of 55 rats pretreated with pyridostigmine showed signs of diarrhea after DFP challenge, while 16 out of 58 rats pretreated with saline were similarly affected (chi square = 11.59, $p < 0.001$). Thus, pyridostigmine pretreatment did counteract the incidence of diarrhea induced be DFP.

Assessment of Physostigmine's Protective Effects.

Several investigators have reported that physostigmine, a centerally acting carbamate anticholinesterase agent, is more effective than pyridostigmine in protecting against the effects of organophosphates (Deshpande et al., 1986; Phlippens et al., 1998; Solana et al., 1990). Therefore, an experiment was conducted to assess the acute protective effect of physostigmine against DFP. Surgery was conducted to implant the transmitters. After one week of recovery the rats were placed into the telemetry chambers for one day to allow adaptation. Then rats were given 4 mg/kg physostigmine or an equivalent volume of saline by gavage. This was followed 30 min later by the DFP (1 mg/kg, i.m.) challenge. Temperature was recorded for a further two hours. It can be seen that the FSL rats initially exhibited a robust hypothermic response to physostigmine itself, whereas the FRL rats did not (Figure 6). Nevertheless, the rats pretreated with saline exhibited greater decreases in temperature at two hr than the rats pretreated with physostigmine, regardless of gender or line (Fig. 6). The shape of the graphs in Fig. 6 is quite distinct from that shown in Fig. 5 for pyridostigmine. Therefore, physostigmine is more effective than pyridostigmine in protecting against the hypothermic effects of DFP.

## Discussion

Almost all of the animals exhibiting diarrhea were either FSL or SD rats, suggesting increased peripheral cholinergic sensitivity in these groups, a conclusion which has been reinforced by other recent studies (Djuric et al., 1995; 1998). These findings are also suggestive of the possibility that pyridostigmine may offer protection against a key peripheral cholinergic symptom induced by DFP. These findings are consistent with other reports of the effectiveness of pyridostigmine in protecting against the peripheral and/or lethal effects of organophosphate nerve agents (e.g. Dirnhuber et al., 1979; French et al., 1979; Walday et al., 1993; Xia et al., 1981).

The greater effects of both CPF and DFP in the pyridostigmine-pretreated rats could be explained by a pharmacokinetic mechanism. Because pyridostigmine is attached to cholinesterase molecules in the periphery, there are fewer binding sites for CPF and DFP. Therefore, more of these agents should penetrate the brain and one could therefore expect a more rapid rate of decline in core body temperature, as seen in Figures 4 & 5. If this hypothesis is correct, then the brain cholinesterase activity may have been more rapidly decreased in the rats that had been pretreated with pyridostigmine and acutely challenged with CPF or DFP. By the time the rats has been sacrificed at two hours after the injections of CPF and DFP, the temperatures were similar, so brain cholinesterase activity may also be similar.

Another mechanism that might be involved is the possibility of adaptive changes to the chronic pyridostigmine treatment which rendered these animals more sensitive to subsequent acute challenges. If muscarinic receptors are elevated following chronic pyridostigmine treatment as they are following neostigmine (Costa et al., 1982), then the

pyridostigmine-treated rats should be more sensitive to the directly acting muscarinic agonist, OXO. The data presented in Figure 6 provide limited support for this hypothesis. The FSL that had been pretreated with pyridostigmine exhibited a more prolonged hypothermic responses, but FRL rats did not. Receptor binding assays will be performed on the brains from these rats in order to determine with there are muscrinic receptor changes as a results of pyridostigmine treatment.

The strain-dependent sensitization to OXO following chronic pyridostigmine treatment provides some clue as to one factor which could have contributed to the develop of the Gulf War Illness. Only rats (FSL) that had an innate hypersensitivity to cholinergic agents became even more sensitive to OXO after pretreatment with pyridostigmine. It is possible, therefore, that those individuals with an innate hypersensitivity to cholinergic agents developed symptoms of the Gulf War Illness after chronic pretreatment with pyridostigmine. It is likely that other factors such as stress and exposure to low levels of organophosphate nerve agents also contributed. However, the present findings suggest that the possibility of a genetic predisposing factor, innate cholinergic hyptersensitivity, needs to be more fully explored. Measurement of serum growth hormone following an acute pyridostigmine challenge, which has been widely used in a variety of human populations (Chaudhuri et al., 1997; Cooney et al., 1997a,b; Ghigo et al., 1993; Lucey et al., 1993; O'Keane et al., 1992, 1994, is one avenue that could be explored.

Acknowledgements

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Table 1

Hypothermic Effects one hour after DFP or CPF Treatment in

Rats Chronically Pretreated with Saline or Pyridistigmine

| STRAIN/SEX | CPF | | DFP | |
|---|---|---|---|---|
| | SAL | PYR | SAL | PYR |
| SD-F | $36.90\pm0.18^{a}$ | $36.07\pm0.08^{a}$ | $37.27\pm0.25^{a}$ | $36.95\pm0.09^{a}$ |
| FSL-F | $37.13\pm0.11^{a}$ | $36.47\pm0.09^{b}$ | $36.29\pm0.34^{b}$ | $35.07\pm0.19^{b}$ |
| FRL-F | $37.85\pm0.04^{b}$ | $37.53\pm0.04^{c}$ | $37.87\pm0.11^{c}$ | $37.32\pm0.07^{c}$ |
| SD-M | $37.18\pm0.05^{a}$ | $36.93\pm0.06^{d}$ | $37.28\pm0.15^{a}$ | $36.62\pm0.15^{a}$ |
| FSL-M | $36.70\pm0.12^{c}$ | $36.83\pm0.09^{d}$ | $36.95\pm0.19^{b}$ | $35.78\pm0.23^{b}$ |
| FRL-M | $37.17\pm0.03^{a}$ | $37.00\pm0.04^{d}$ | $37.70\pm0.07^{c}$ | $37.20\pm0.08^{c}$ |
| F (Treatment) = | 14.52, $p < 0.01$ | | 11.22, $p < 0.01$ | |
| F (Strain/Sex) | 81.47, $p < 0.001$ | | 109.76, $p < 0.001$ | |

Groups with different letters are significantly different from each other, Tukey's test..

Table 2

Hypothermic Effects two hours after DFP or CPF Treatment in

Rats Chronically Pretreated with Saline or Pyridistigmine

| STRAIN/SEX | CPF | | DFP | |
|---|---|---|---|---|
| | SAL | PYR | SAL | PYR |
| SD-F | $35.20\pm0.08^{a}$ | $35.34\pm0.06^{a}$ | $35.43\pm0.09^{a}$ | $36.10\pm0.09^{a}$ |
| FSL-F | $36.07\pm0.07^{b}$ | $35.64\pm0.06^{a}$ | $34.50\pm0.11^{b}$ | $34.53\pm0.06^{b}$ |
| FRL-F | $37.41\pm0.10^{c}$ | $37.47\pm0.06^{b}$ | $37.26\pm0.06^{c}$ | $37.16\pm0.05^{c}$ |
| SD-M | $36.10\pm0.12^{b}$ | $35.78\pm0.12^{a}$ | $36.07+0.07^{d}$ | $35.73\pm0.10^{a}$ |
| FSL-M | $35.64\pm0.11^{a,b}$ | $35.94\pm0.07^{a}$ | $35.23\pm0.12^{a}$ | $34.56\pm0.04^{b}$ |
| FRL-M | $37.03\pm0.06^{c}$ | $36.87\pm0.08^{c}$ | $36.94\pm0.06^{c}$ | $36.69\pm0.10^{d}$ |
| F(treatment) = | 0.45, NS | | 0.75, NS | |
| F (strain/sex) | 481.36, $p < 0.001$ | | 667.5, $p < 0.001$ | |

Groups with different letters are significantly different from each other, Tukey's test..

## FIGURE CAPTIONS

Figure 1. Strain and Gender-Dependent Effects of Oxotremorine in FSL, FRL and SD Rats. After the recording of baseline temperatures, rats were injected sc with a mixture of 2 mg/kg methyl atropine nitrate and 0.2 mg/kg oxotremorine sesquifumarate. The scores represent the mean decrease from baseline temperature (°C) for 25-30 rats at 60 min after the injection. Different letters indicate that the groups are significantly different from each other according to Tukey's tests.

Figure 2A-F. Changes in Telemetrically Monitored Temperature in FSL, FRL and SD Rats following Chronic Saline or Pyridostigmine Treatment and Acute Saline Challenges. Rats were chronically treated by gavage with pyridostigmine (12 mg/kg) or saline for 14 days. The saline challenge (gavage or intramuscular) was given 30 min after the $14^{th}$ treatment and temperature was monitored for a further 2 hr. The data represent the mean temperatures for 8-10 animals. Standard errors are omitted for clarity.

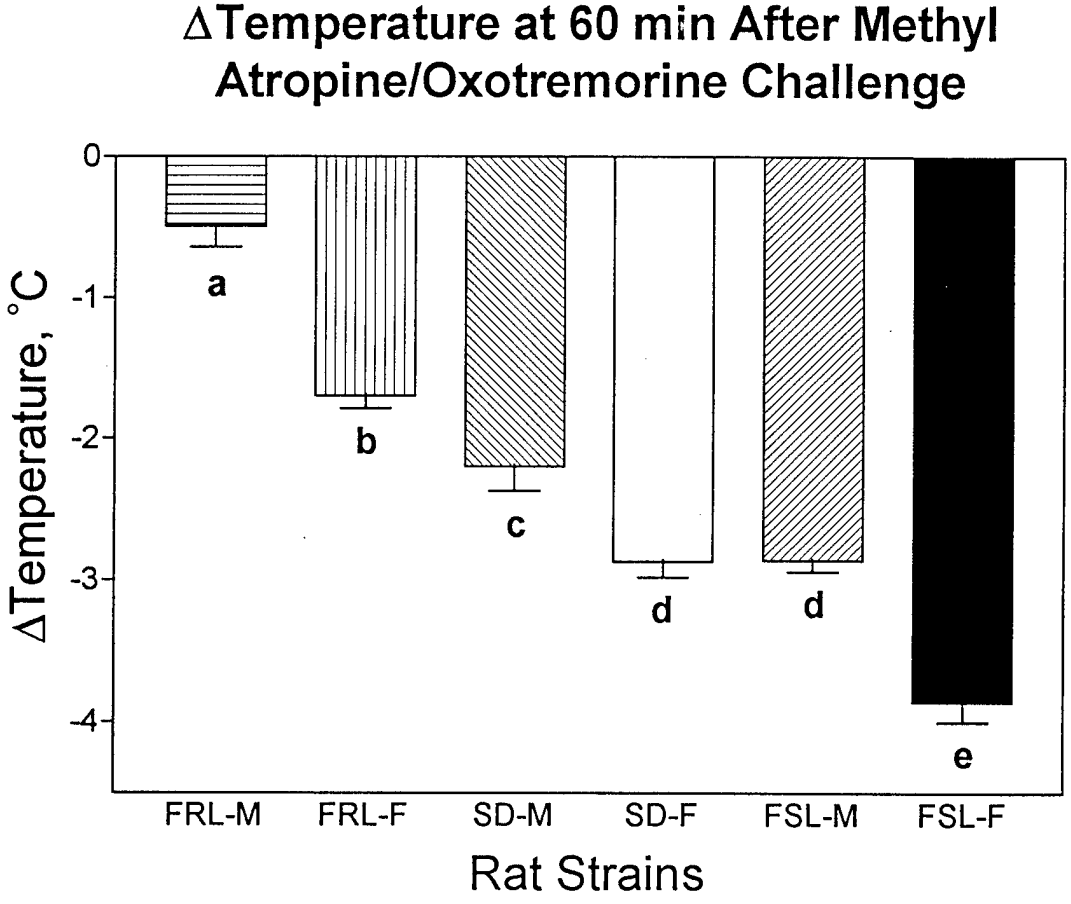
Figure 3A-F. Changes in Telemetrically Monitored Temperature in FSL, FRL and SD Rats following Chronic Saline or Pyridostigmine Treatment and Acute Chlorpyrifos (CPF) Challenges. Rats were chronically treated by gavage with pyridostigmine (12 mg/kg) or saline for 14 days. The CPF challenge (60 mg/kg by gavage) was given 30 min after the $14^{th}$ treatment and temperature was monitored for a further 2 hr. The data represent the mean temperatures for 8-10 animals. Standard errors are omitted for clarity.

Figure 4A-F. Changes in Telemetrically Monitored Temperature in FSL, FRL and SD Rats following Chronic Saline or Pyridostigmine Treatment and Acute Diisopropylfluorophosphate (DFP) Challenges. Rats were chronically treated by gavage with pyridostigmine (12 mg/kg) or saline for 14 days. The DFP challenge (1 mg/kg, s.c.)

was given 30 min after the 14th treatment and temperature was monitored for a further 2 hr. The data represent the mean temperatures for 8-10 animals. Standard errors are omitted for clarity.

Figure 5A-F. Changes in Telemetrically Monitored Temperature in FSL, FRL and SD Rats following Chronic Saline or Pyridostigmine Treatment and Acute Oxotremorine (OXO) Challenges. Rats were chronically treated by gavage with pyridostigmine (12 mg/kg) or saline for 10 days. The OXO challenge (0.2 mg/kg with 2 mg/kg methyl atropine, s.c.) was given 30 min after the 10th treatment and temperature was monitored for a further 6 hr. The data represent the mean temperatures for 8-10 animals. Standard errors are omitted for clarity.

Figure 6. Changes in Telemetrically Monitored Temperature in FSL and FRL rats following Acute Pretreatment with Saline or Physostigmine and Acute DFP challenges. Rats were pretreated with physostigmine (4 mg/kg) by gavage. The DFP challenge (1.0 mg/kg, i.m.) was given 30 min after the physostigmine pretreatment. Temperature was monitored for two hours after the DFP treatment. The data represent the mean temperatures for 8-10 animals. Standard errors are omitted for clarity.


ΔTemperature at 60 min After Methyl Atropine/Oxotremorine Challenge
ΔTemperature, °C
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-1
-2
-3
-4
a
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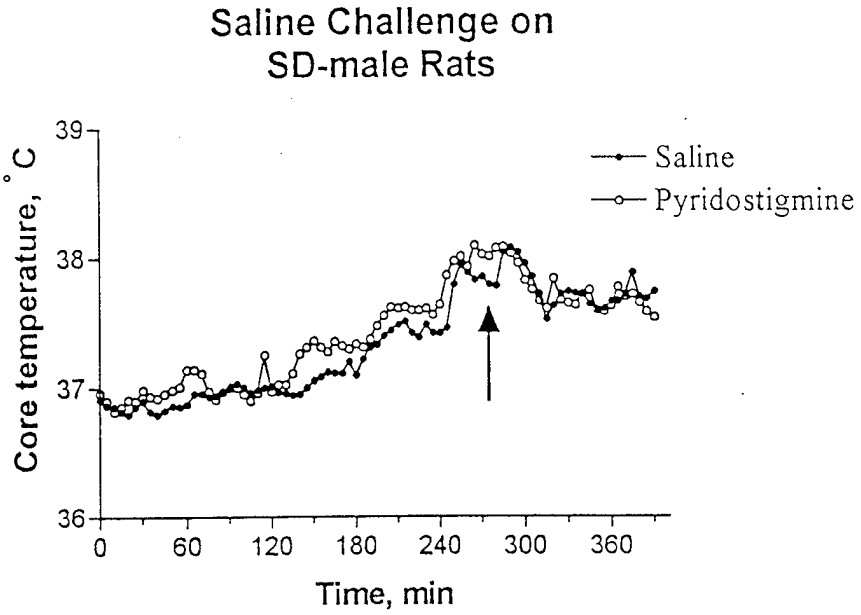
d
e
FRL-M
FRL-F
SD-M
SD-F
FSL-M
FSL-F
Rat Strains

A


Saline Challenge on
SD-male Rats
Saline
Pyridostigmine
Core temperature, °C
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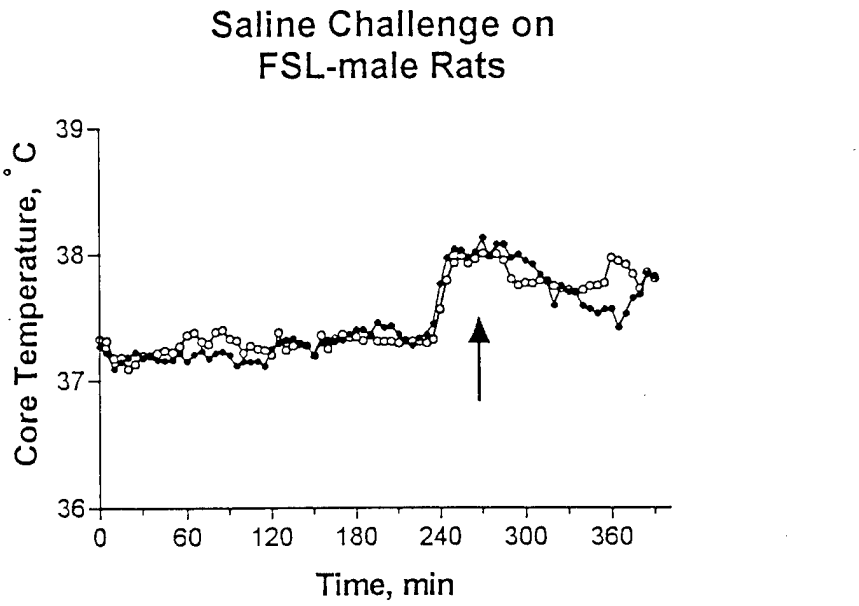
240
300
360
Time, min

B


Saline Challenge on
FSL-male Rats
Core Temperature, °C
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Time, min

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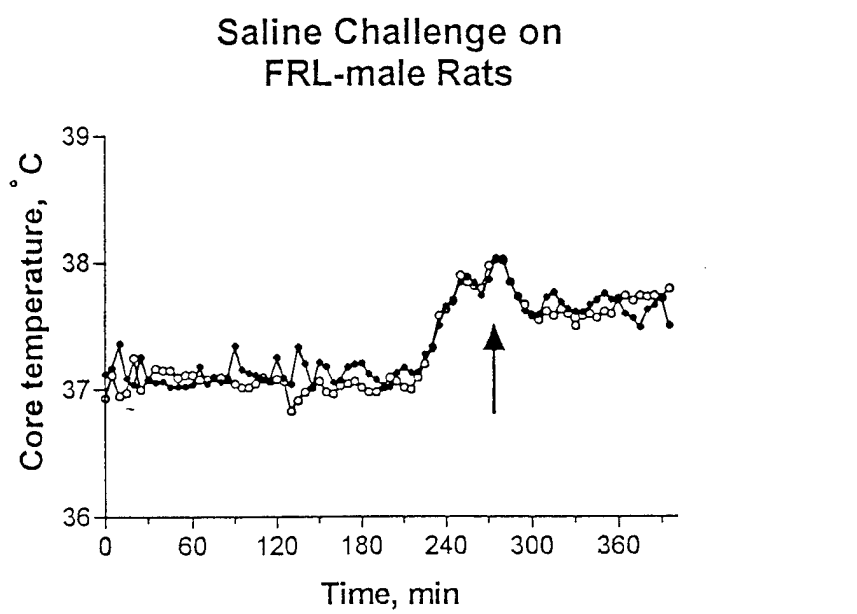
Saline Challenge on
FRL-male Rats
Core temperature, °C
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Time, min

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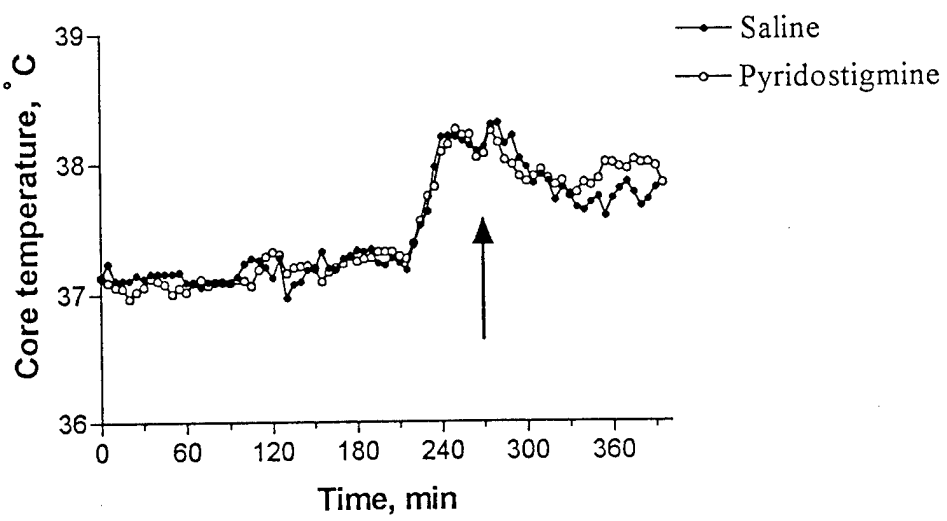
Saline Challenge on
SD-female Rats
Saline
Pyridostigmine
Core temperature, °C
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Time, min

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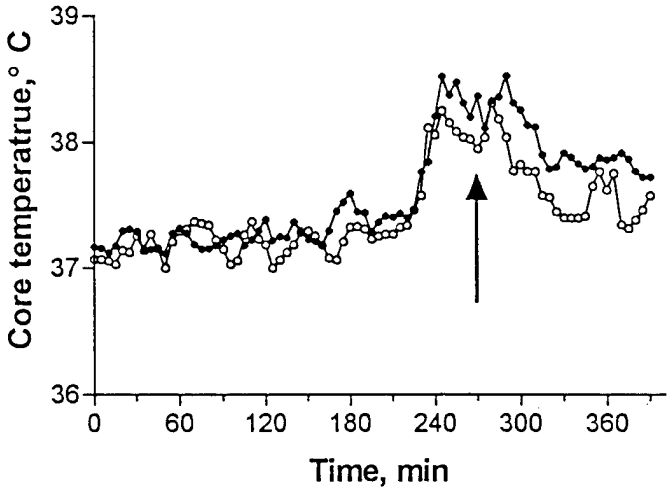
Saline Challenge on
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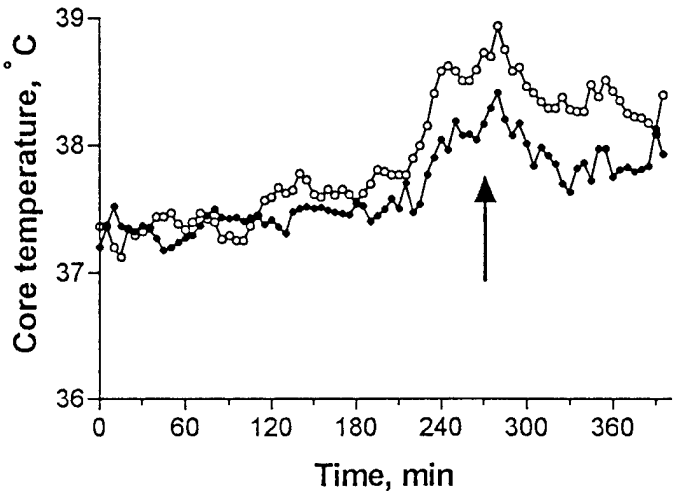
Saline Challenge on
FRL-female Rats
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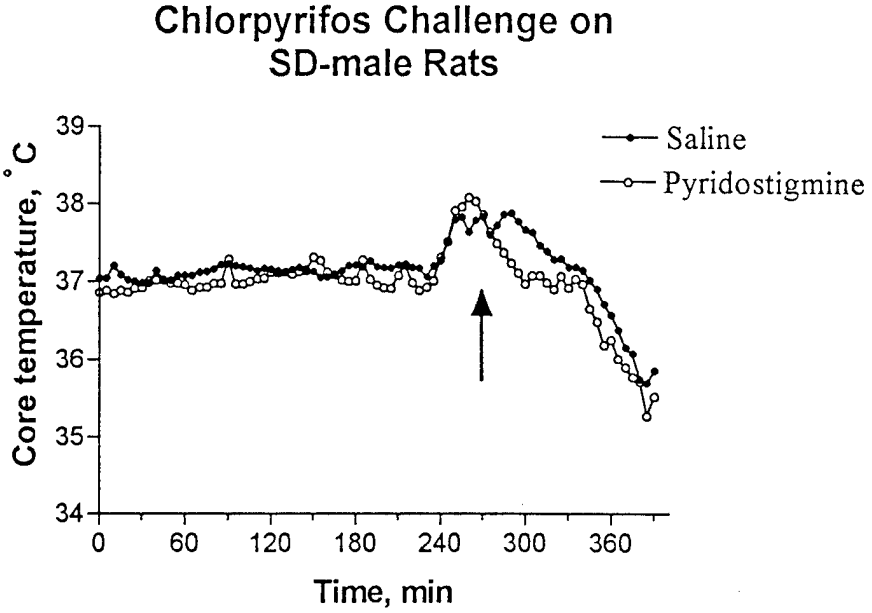
Chlorpyrifos Challenge on
SD-male Rats
Saline
Pyridostigmine
Core temperature, °C
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Time, min

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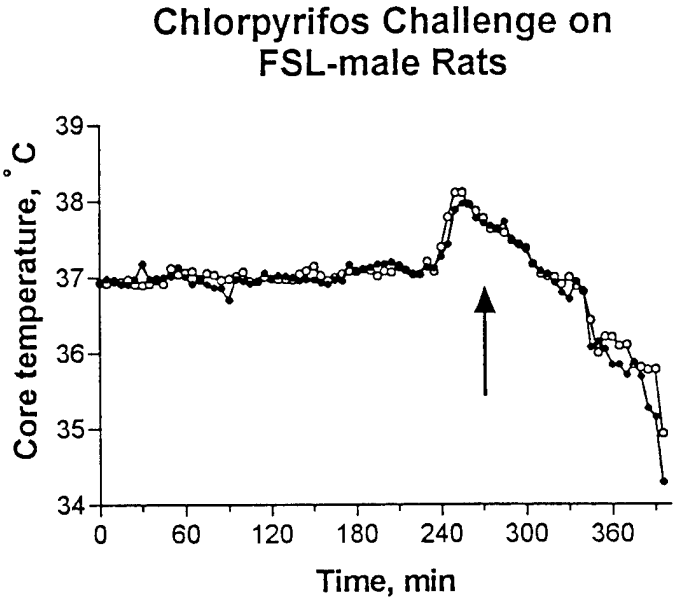
Chlorpyrifos Challenge on
FSL-male Rats
Core temperature, °C
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Time, min

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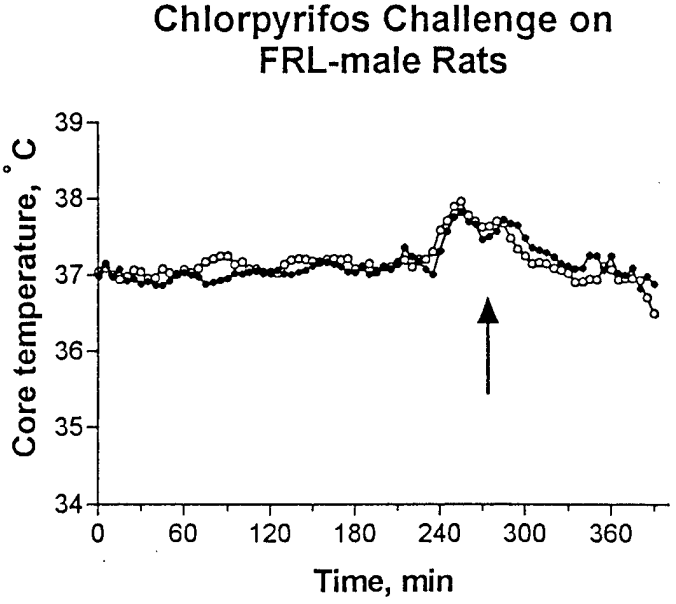
Chlorpyrifos Challenge on
FRL-male Rats
Core temperature, °C
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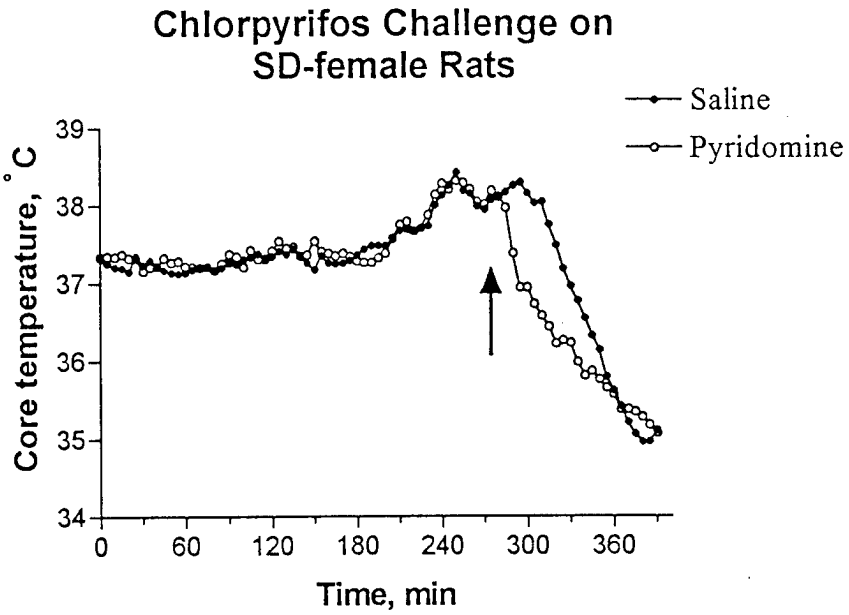
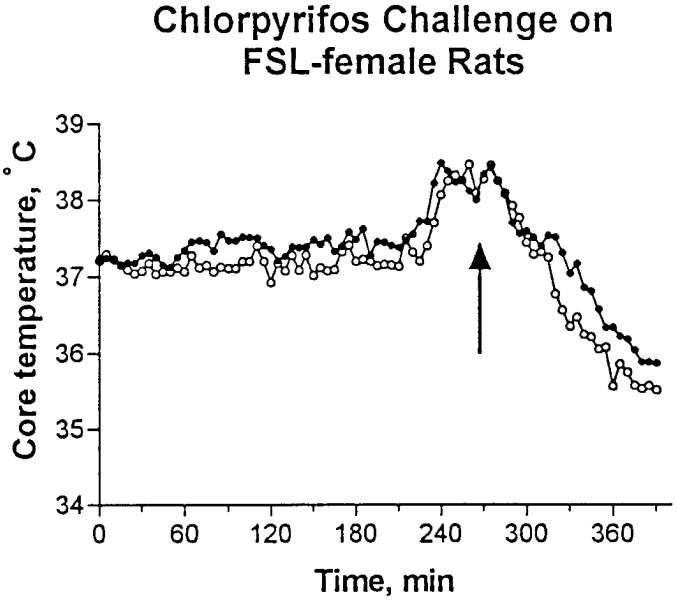
360
Time, min

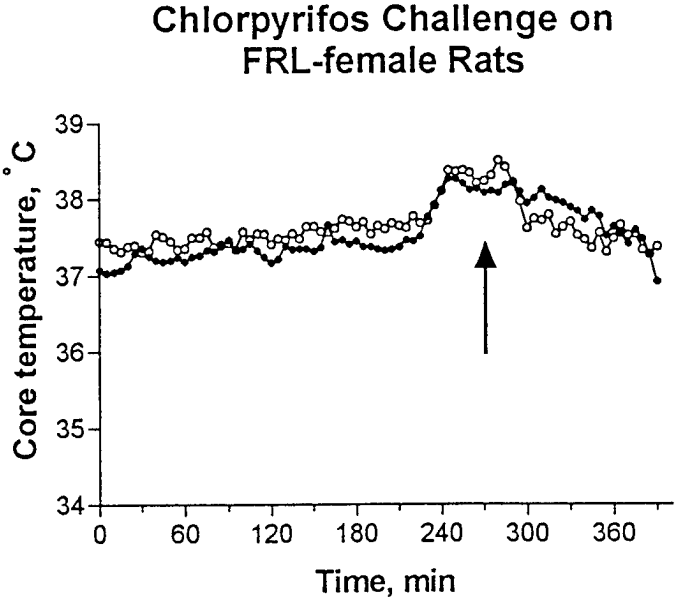
D


Chlorpyrifos Challenge on
SD-female Rats
Saline
Pyridomine
Core temperature, °C
39
38
37
36
35
34
0
60
120
180
240
300
360
Time, min

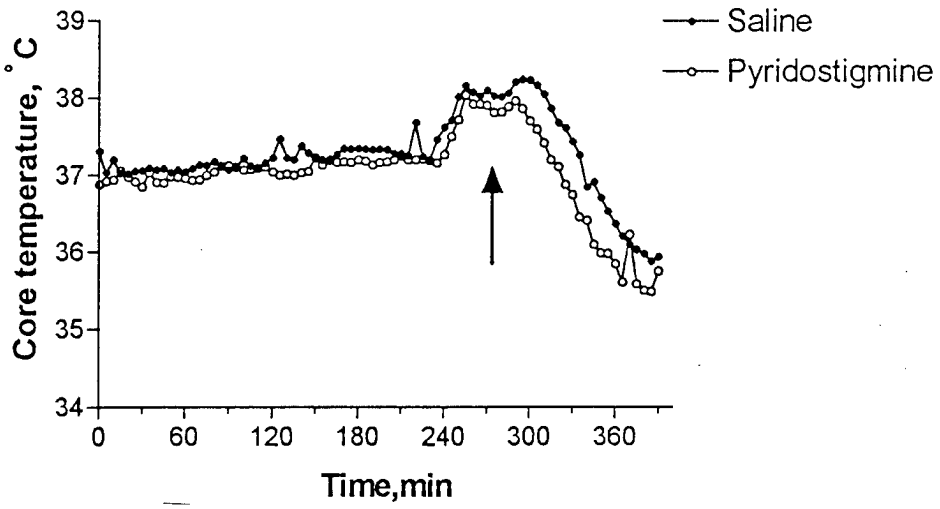
E


Chlorpyrifos Challenge on
FSL-female Rats
Core temperature, °C
39
38
37
36
35
34
0
60
120
180
240
300
360
Time, min

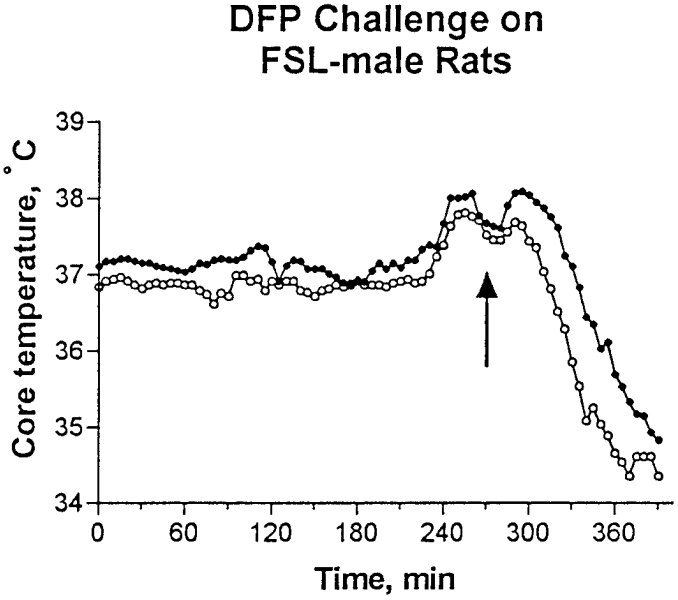
F


Chlorpyrifos Challenge on
FRL-female Rats
Core temperature, °C
39
38
37
36
35
34
0
60
120
180
240
300
360
Time, min

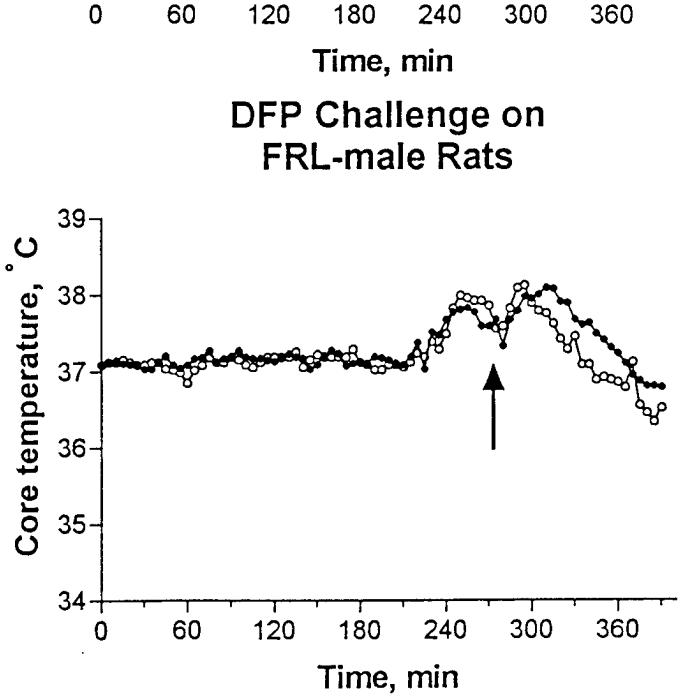
A


DFP Challenge on
SD-male Rats
Saline
Pyridostigmine
Core temperature, °C
39
38
37
36
35
34
0
60
120
180
240
300
360
Time,min

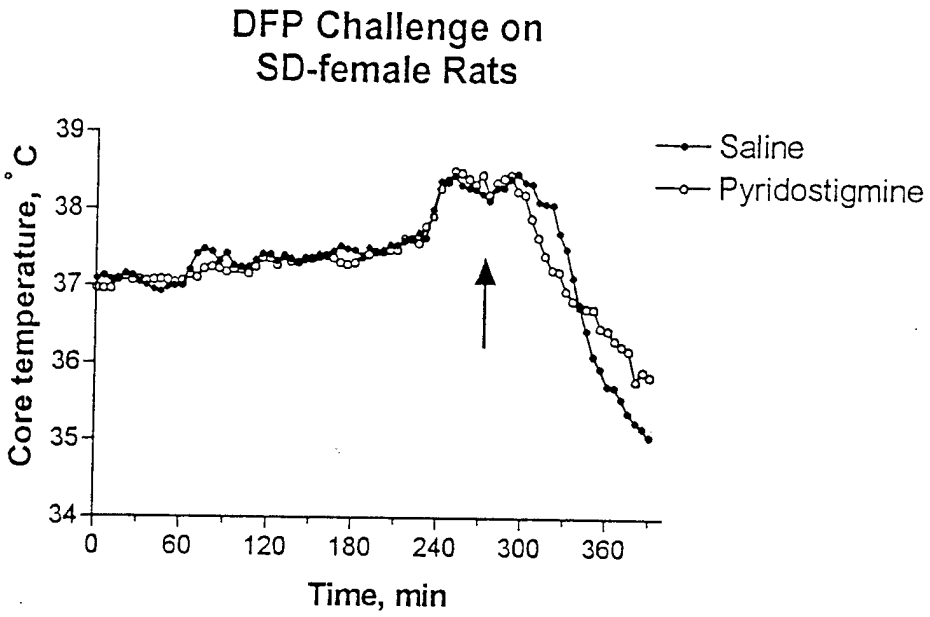
B


DFP Challenge on
FSL-male Rats
Core temperature, °C
39
38
37
36
35
34
0
60
120
180
240
300
360
Time, min

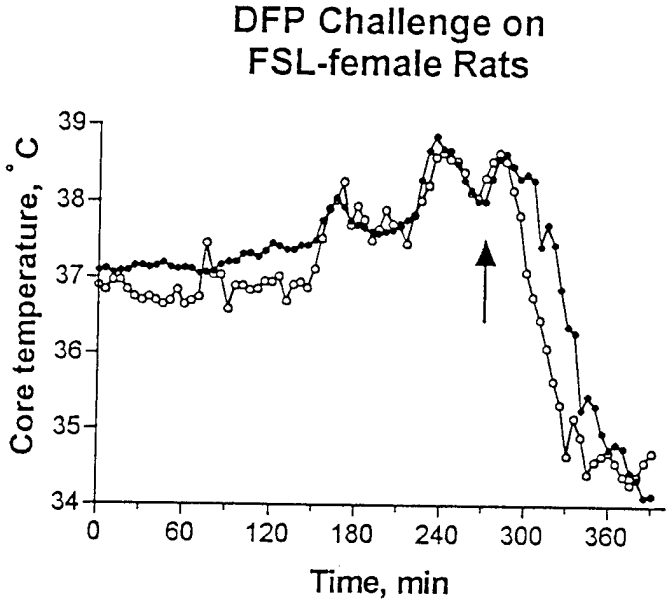
C


DFP Challenge on
FRL-male Rats
Core temperature, °C
39
38
37
36
35
34
0
60
120
180
240
300
360
Time, min

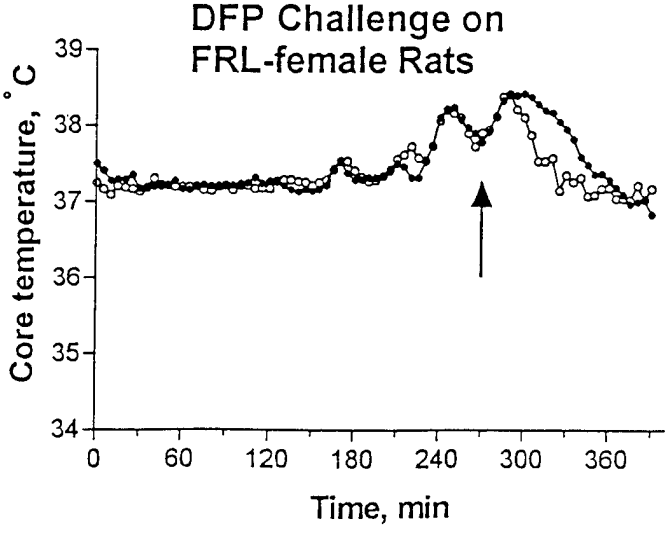
D


DFP Challenge on
SD-female Rats
Saline
Pyridostigmine
Core temperature, °C
39
38
37
36
35
34
0
60
120
180
240
300
360
Time, min

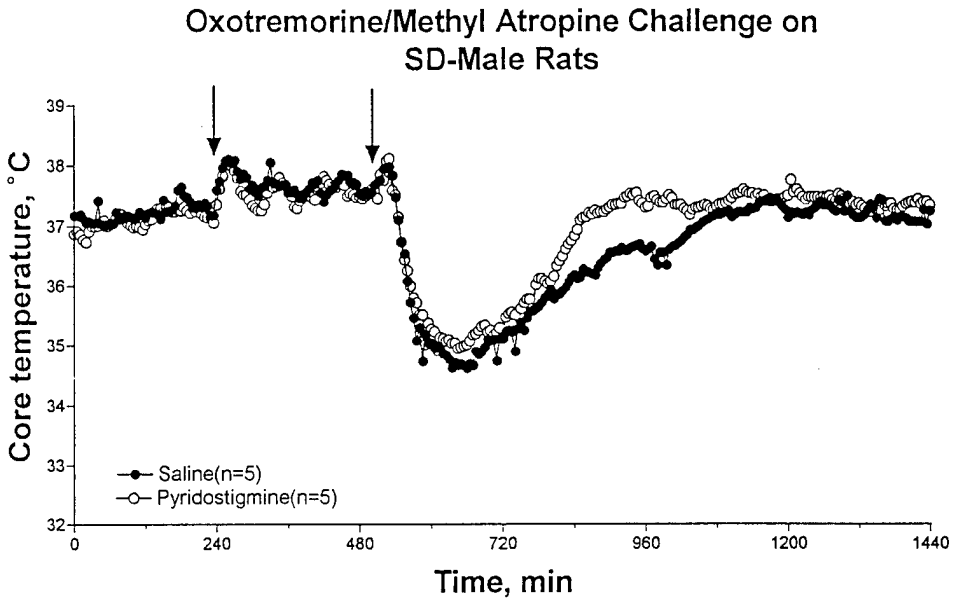
E


DFP Challenge on
FSL-female Rats
Core temperature, °C
39
38
37
36
35
34
0
60
120
180
240
300
360
Time, min

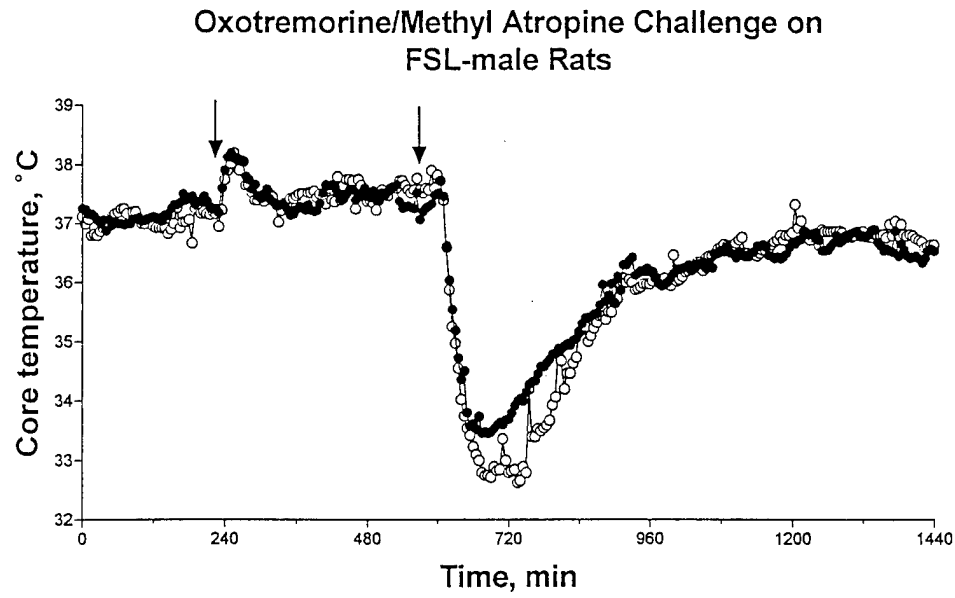
F


DFP Challenge on
FRL-female Rats
Core temperature, °C
39
38
37
36
35
34
0
60
120
180
240
300
360
Time, min

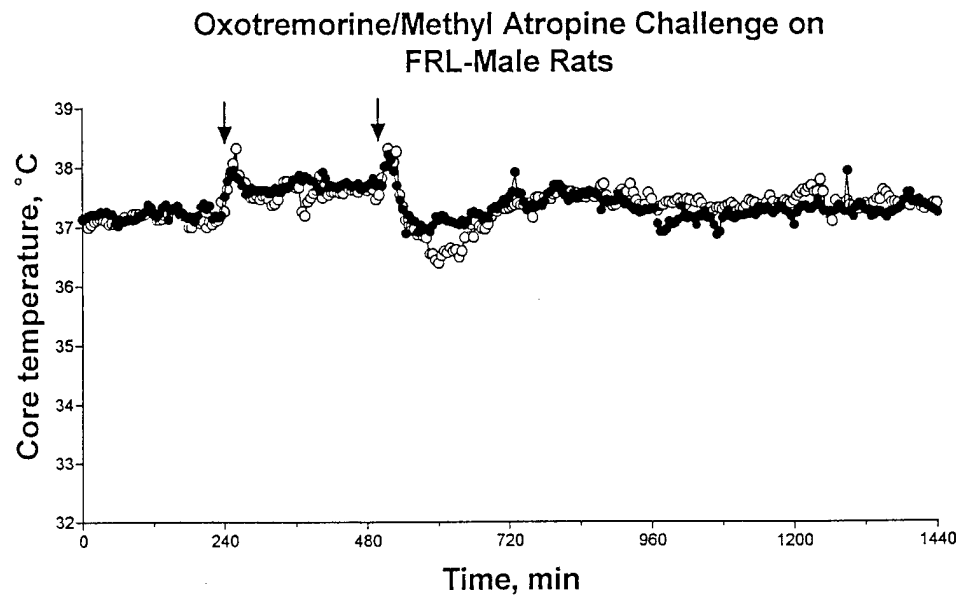
A


Oxotremorine/Methyl Atropine Challenge on
SD-Male Rats
Core temperature, °C
Time, min
Saline(n=5)
Pyridostigmine(n=5)
39
38
37
36
35
34
33
32
0
240
480
720
960
1200
1440

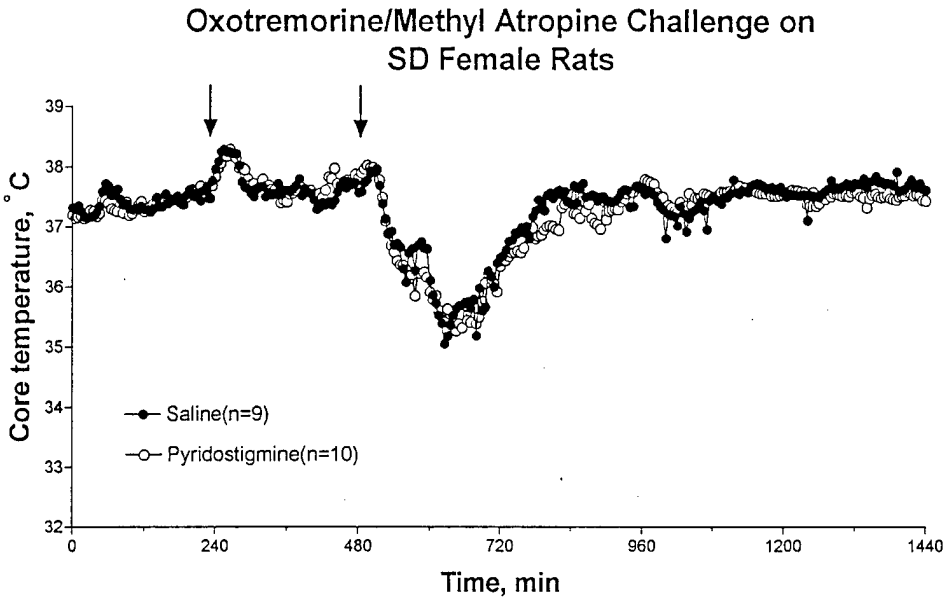
B


Oxotremorine/Methyl Atropine Challenge on
FSL-male Rats
Core temperature, °C
Time, min
39
38
37
36
35
34
33
32
0
240
480
720
960
1200
1440

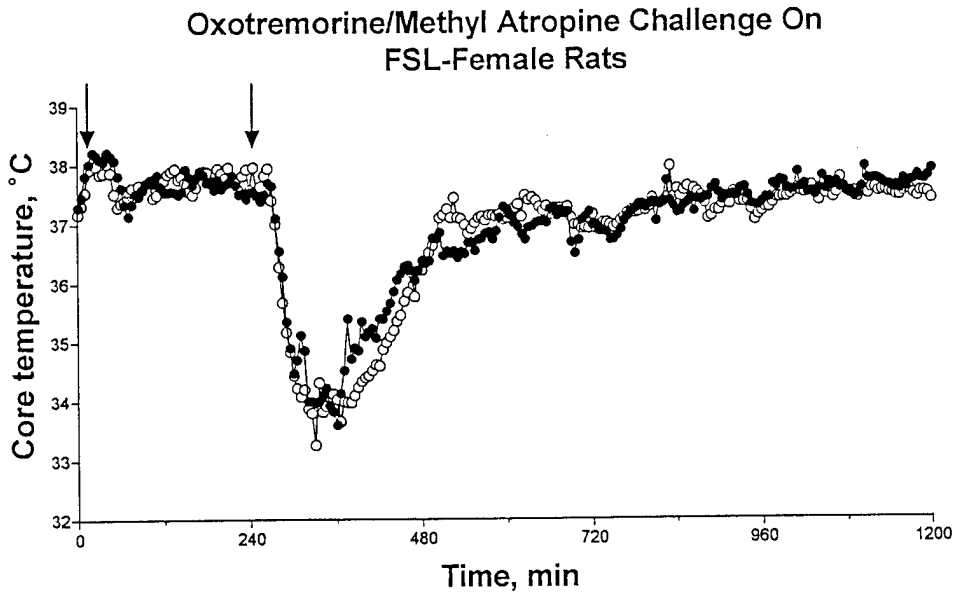
C


Oxotremorine/Methyl Atropine Challenge on
FRL-Male Rats
Core temperature, °C
Time, min
39
38
37
36
35
34
33
32
0
240
480
720
960
1200
1440

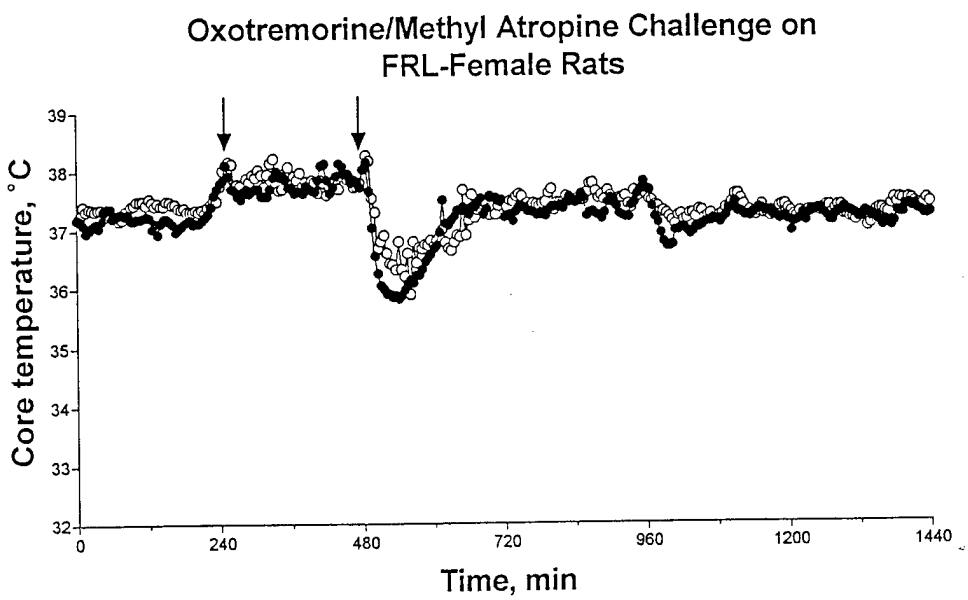
D


Oxotremorine/Methyl Atropine Challenge on
SD Female Rats
Core temperature, °C
Saline(n=9)
Pyridostigmine(n=10)
Time, min

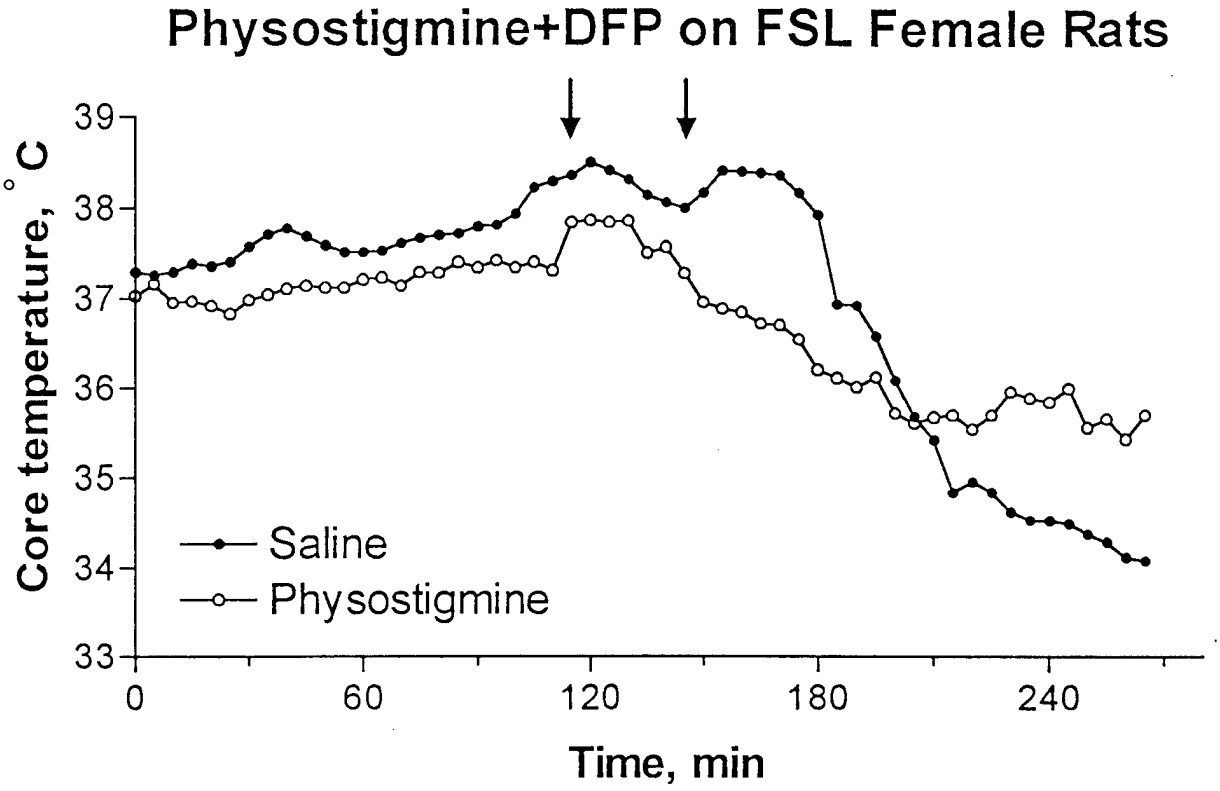
E


Oxotremorine/Methyl Atropine Challenge On
FSL-Female Rats
Core temperature, °C
Time, min

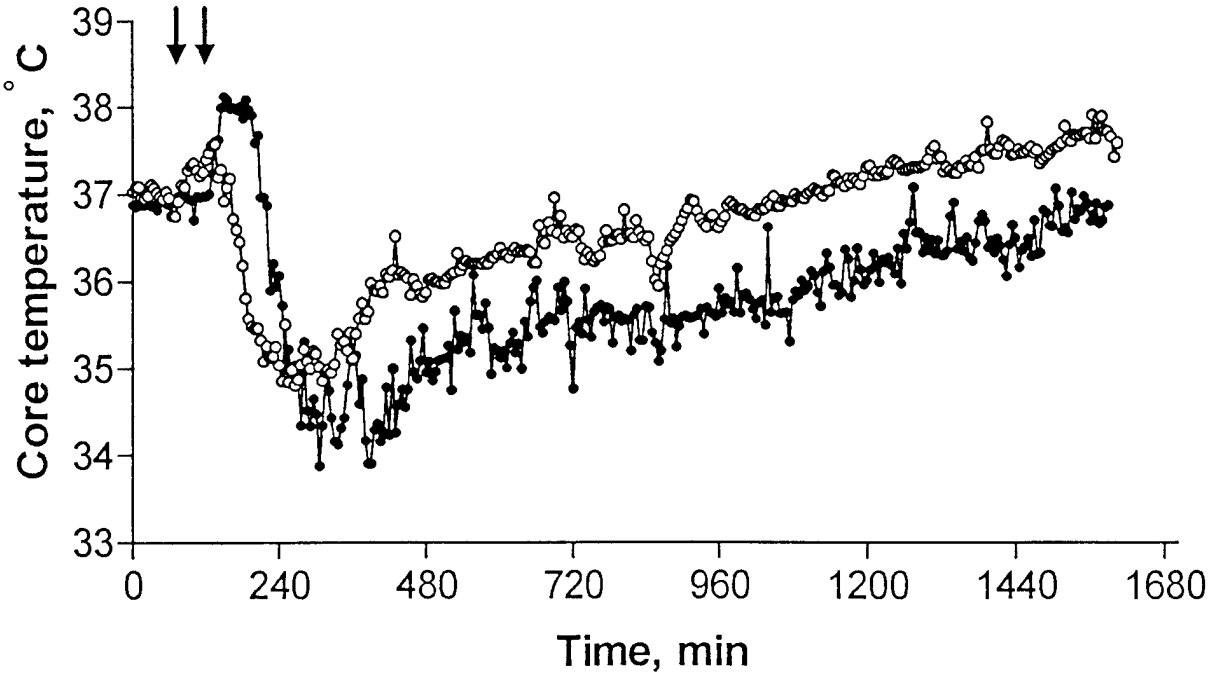
F


Oxotremorine/Methyl Atropine Challenge on
FRL-Female Rats
Core temperature, °C
Time, min

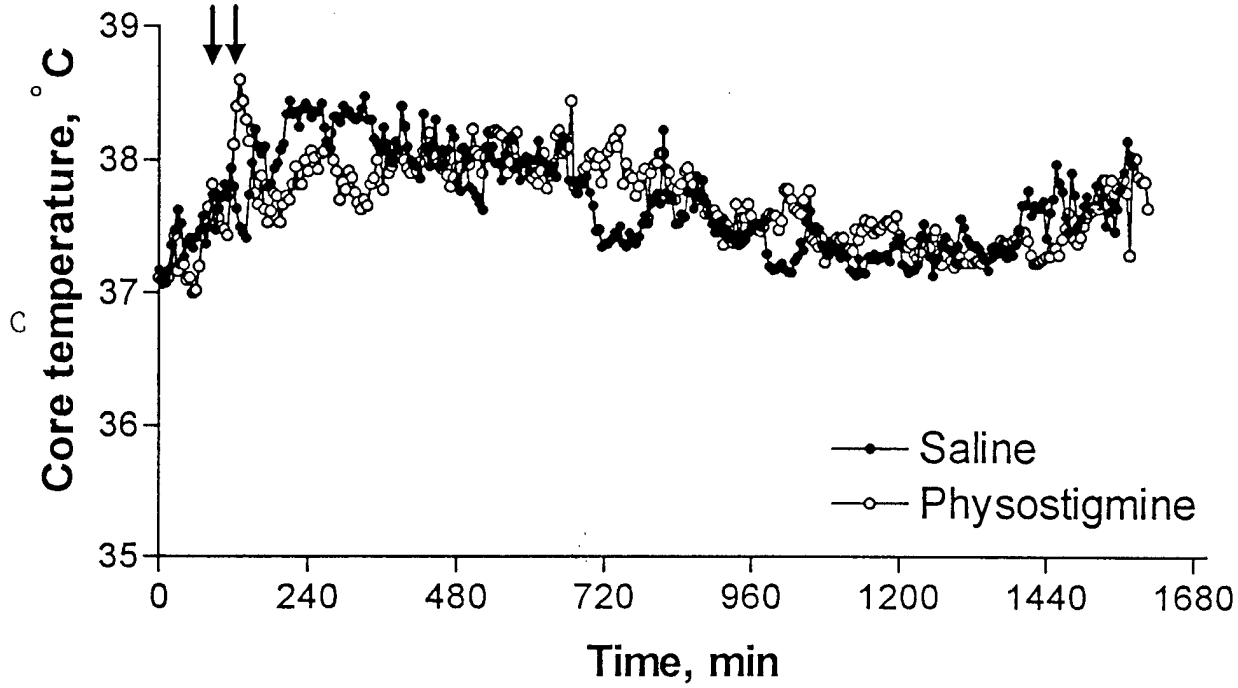
A


Physostigmine+DFP on FSL Female Rats
Core temperature, °C
39
38
37
36
35
34
33
0
60
120
180
240
Time, min
Saline
Physostigmine

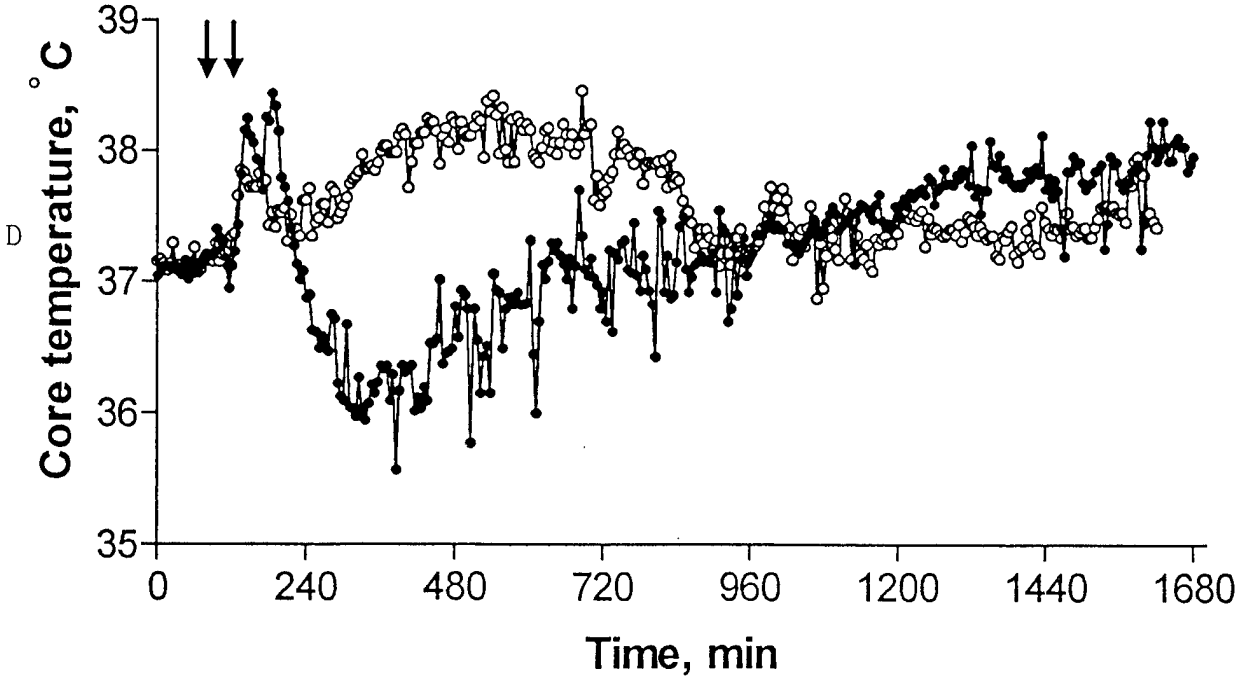
b


Physostigmine+DFP on FSL Male Rats
(24hr)
Core temperature, °C
39
38
37
36
35
34
33
0
240
480
720
960
1200
1440
1680
Time, min

C


Physostigmine+DFP on FRL Female Rats
(24hr)
Core temperature, °C
39
38
37
36
35
0
240
480
720
960
1200
1440
1680
Time, min
Saline
Physostigmine

D


Physostigmine+DFP on FRL Male Rats
(24hr)
Core temperature, °C
39
38
37
36
35
0
240
480
720
960
1200
1440
1680
Time, min

728.15
SPATIAL LEARNING AND CA1 HIPPOCAMPAL SYNAPTIC PLASTICITY IN MDX AND MDX3CV MICE, MODELS OF DUCHENNE MUSCULAR DYSTROPHY. C. Vaillend[1], J.-M. Billard[2]*, P. Dutar[2], and A. Ungerer[1]. [1] URA-CNRS 1295, ULP, Strasbourg, France; [2]INSERM U161, Paris, France.

X-linked Duchenne muscular dystrophy (DMD) is frequently associated with a non-progressive cognitive defect attributed to the absence of 427 kDa dystrophin (Dp427) in the brain of DMD patients, or to altered expression of other C-terminal isoforms of brain dystrophin, such as Dp140 or Dp71. In this work, we investigated spatial learning and synaptic plasticity in both the mdx mouse, which lacks Dp427, and the new mdx3cv mutant, which lacks Dp427, Dp140, and Dp71. Mice were trained during 9 days in a spatial discrimination task in an 8-arm radial maze with 4 baited arms, to evaluate working (WM) and reference memory (RM) performance. Synaptic strengthening was studied in CA1 hippocampal slices from both strains, using paired-pulse facilitation (PPF) and long-term potentiation (LTP) paradigms. In the radial-maze task, the number of visits to non-baited arms and of returns into a previously visited arm did not differ over training between mdx and matched controls, or between mdx3cv and controls, indicating that WM and RM were not significantly impaired in these two mutants. The latencies to visit the first arm were two-fold longer in mdx3cv than in controls on the first learning session, suggesting enhanced emotional reactivity in mdx3cv, when compared to mdx mice. Electrophysiological recordings revealed that mdx, but not mdx3cv mice, have increased post-tetanic potentiation when compared to controls, which could be linked to calcium-specific regulatory defects. Both mutants showed normal PPF for various interstimulus intervals and LTP levels comparable to that of controls, which suggest that these forms of synaptic plasticity are not altered in the CA1 hippocampal field of dystrophin-deficient mice.

Supported by A. F. M. (Association Française contre les Myopathies, France).

728.16
TIME COURSE OF THALAMO-CORTICAL SYNCHRONIZATION DURING ABSENCE SEIZURES IN A GENETIC RAT MODEL.
T. Seidenbecher*, R. Staak, S. Staak and H.-C. Pape. Institute of Physiology of Medical Academy, Otto-v.-Guericke University Magdeburg 39120, Germany

While the general involvement of thalamo-cortical circuits in the genesis of generalized spike-and-wave discharges (SWD) is well documented, the exact time course of recruitment of cellular activities in various thalamic and cortical regions remains largely unknown. In this study, bilateral electroencephalographic (EEG) and multi-unit activity was recorded simultaneously during generalized SWD in anesthetized Genetic Absence Epilepsy Rats from Strasbourg (GAERS). Acute experiments were performed under fentanyl-droperidol anesthesia. All investigated animals developed generalized 7-11 Hz SWD spontaneously. Multi-unit activity was recorded in the somatosensory cortex and different thalamic nuclei [ventroposterolateral (VPL), ventroposteromedial (VPM), ventrolateral (VL), anteroventral (AVVL), rostral (rRT) and caudal nucleus reticularis (cRT)]. The negative peak of the spike component of the SWD was used as trigger for calculation of stimulus histograms. Neurons of cortex, VPL, VPM, VL and RT nuclei showed a spike-locked synchronized excitation and a wave-locked depression during absence seizures. By contrast, neuronal activity of the AVVL showed a spike related depression followed by a wave-locked rebound activity. Maximum firing of neurons of all recorded structures (except AVVL) preceded the peak of the spike. Peak latencies of unit activities related to the spike component of the SWD showed statistically significant differences. Rhythmical neuronal activity of the cortex precedes neuronal discharges of thalamic neurons and was evident up to 1.9 seconds before the EEG correlates.

We conclude that neuronal activity in the somatosensory cortical and thalamic regions are highly synchronized with the spike phase of SWD, while the activity in the anterior thalamic nucleus is depressed. The present data favor the leading role of the cortex as a possible pacemaker in the generation of SWD in GAERS.

728.17
GENETIC DISSECTION OF THE SIGNALS THAT INDUCE SYNAPTIC REORGANIZATION. P.E. Schauwecker* and O. Steward. Dept. of Neuroscience, Univ. of Virginia, Charlottesville, VA 22908.

We have recently discovered that certain strains of inbred mice are virtually invulnerable to kainic acid (KA)-induced excitotoxicity. Administration of KA induces seizures and epileptiform activity. The seizures cause excitotoxic cell death of pyramidal neurons in CA1 and CA3 subfields and dentate hilar neurons and subsequent synaptic rearrangement within the dentate gyrus. Since it is not known whether kainate induced seizures themselves or cellular damage are triggers for synaptic rearrangment, our identification of strains that have seizures but no cell death provides a means for relating molecular processes to synaptic remodelling. Following KA administration, both strains exhibited seizures of a similar duration and intensity and showed similar spatiotemporal patterns of 2-deoxyglucose (2-DG) uptake within the hippocampus. However, invulnerable mice showed a greater increase in 2-DG uptake at high and intermediate doses of KA. In contrast, invulnerable mice exhibited acute, transient increases in c-fos immunoreactivity in hippocampal neurons, while vulnerable mice demonstrated prolonged increases in c-fos immunoreactivity in areas where collateral sprouting occurs. Evidence of sprouting, as detected by increased histochemical staining of mossy fibers and septohippocampal projections, was only evident in strains vulnerable to KA. These findings provide strong evidence that a transient activation of c-fos may be seizure-related while protracted induction of c-fos is related to impending cell death and neural plasticity. The identification of strains that elicit different cellular cascades based on their vulnerability to KA provides evidence for the hypothesis that synaptic reorganization following KA occurs in response to cellular signals associated with cell death. Supported by NS09972-02 to P.E.S. and NS 29875 to O.S.

728.18
GENETIC SENSITIVITY TO COCAINE-KINDLED SEIZURES. G.G. Smith, G.T. Golden, T.N. Ferraro, R.L. Snyder, T.A. Hare*, W.H. Berrettini. Department of Veterans Affairs Medical Center, Coatesville, PA 19320 and Thomas Jefferson University, Philadelphia, PA 19107

Pharmacological kindling describes the development of increased seizure activity following the repeated administration of various excitatory compounds. To delineate genetic factors that may influence susceptibility to pharmacological kindling we first studied the development of cocaine-kindled seizures in two inbred mouse strains: C57, resistant to acute cocaine-induced seizures; and SJL, susceptible to acute cocaine-induced seizures. Two dosing regimes (ip) were used : 40mg/kg/d (n=6/strain) or 50 mg/kg/d (n=10/strain). Results showed large differences in susceptibility to kindled seizures. After 20 daily cocaine injections (40mg/kg), cumulatively 50% of SJL mice showed seizures in contrast to C57 mice which all remained seizure free. At the 50mg/kg/d dose, cumulatively 40% of C57 mice exhibited kindled seizure activity by day 5; however, this effect was not long lasting as no seizures were observed on days 6 -10. In contrast, SJL mice showed cumulatively 100% seizures by day 3 and this effect persisted throughout the remainder of the 10 day treatment period. Seventy-two hours following the last daily cocaine injection, the same mice were challenged with either 70mg/kg (for mice kindled at 50mg/daily dose) or 60mg/kg (for mice kindled at 40mg/daily dose) and the number of mice in each strain exhibiting seizures, latency to first seizure and total time in seizure activity in response to the 72 hour cocaine challenge dose were monitored. ANOVAs for cocaine challenge measures showed significant strain ($p < 0.001$), cocaine dose ($p < 0.001$) and strain X cocaine dose interaction ($p < 0.002$) effects for total time in seizure activity and a significant strain effect ($p < 0.004$) for seizure latency. Results indicate that similar to their relative sensitivities to acute cocaine seizures, C57 mice are resistant to cocaine-kindled seizures whereas SJL mice are sensitive. Determination of the amounts of genetic versus environmental influences in this model may lead to further studies to elucidate the nature of specific genes involved in kindled seizures and epilepsy. (Supported by VHA)

728.19
DIFFERENTIAL SENSITIVITY TO ACUTE COCAINE-INDUCED SEIZURES IN INBRED STRAINS OF MICE. G.T. Golden*, T.N. Ferraro, G.G. Smith, N.L. Jones, W.H. Berrettini. Department of Veterans Affairs Medical Center, Coatesville, PA 19320 and Thomas Jefferson University, Philadelphia, PA

Seizures are a major factor implicated in cocaine related deaths. In the present study mature mice from six inbred strains were tested for susceptibility to seizures induced by an acute injection of cocaine. A total of 257 mice (C57BL/6J n=67, DBA/2J n=48, BALB/cByJ n=48, SJL/J n=63, NZW/LacJ n=21, A/J n=10) were tested using ip injection of either 60 or 70 mg/kg of cocaine HCl. Results showed the rank order (highest to lowest sensitivity) to acute cocaine-induced seizures is A/J = SJL > BALB > DBA = NZW > C57. Mice from the C57 strain were the most resistant and mice from the SJL and A/J strains were the most sensitive.

PERCENTAGE OF MICE WITH BEHAVIORAL SEIZURES

| COCAINE DOSE | C57 MALE | C57 FEM | SJL MALE | SJL FEM | A/J MALE |
|---|---|---|---|---|---|
| 60 mg/kg | 0% | 0% | 57% | 30% | 80% |
| 70 mg/kg | 0% | 17% | 71% | 100% | 80% |

A chi square analysis for strain differences in seizure expression using all six strains (collapsed across sex) was significant at both the 60 mg/kg (Chi Square=28.0, p<.0001) and 70 mg/kg (Chi Square=34.02, p<.0001) cocaine dose . In addition to monitoring the number of mice from each strain exhibiting seizures, latency from time of cocaine injection to initiation of seizure activity was monitored for all animals and analyzed by strain and sex. A two factor ANOVA revealed significant (p<.0001) strain and cocaine dose main effects for seizure latency. Conservative post hoc comparisons revealed significant differences between most strains with the exception of C57 vs NZW, DBA vs BALB, DBA vs NZW, BALB vs NZW and SJL vs A/J. Differences in strain sensitivity to cocaine-induced seizures likely reflect both genetic and environmental influences with their relative magnitudes and sources yet to be determined. In particular there is need to assess possible strain specific pharmacokinetic variables related to cocaine administration. (Supported by VHA)

728.20
STRAIN- AND GENDER-DEPENDENT EFFECTS OF OXOTREMORINE AND PYRIDOSTIGMINE. D.H. Overstreet*, Y. Yang, M. Hamedi, D.S. Janowsky and Amir H. Rezvani, Skipper Bowles Ctr. for Alcohol Studies, UNC, Chapel Hill, NC 27599-7178.

Pyridostigmine (PYR) was given to many participants in the Gulf War as a protection against exposure to nerve gases and some individuals reported side effects. To test the hypothesis that a subset of individuals may have been genetically supersensitive, the effects of PYR were determined in both genders of three strains of rats: The Flinders Sensitive Line (FSL), the Flinders Resistant Line (FRL) and Sprague-Dawley (SD). Rats were first challenged with a mixture of methyl atropine (2 mg/kg) and oxotremorine (OX - 0.2 mg/kg) and temperatures telemetrically monitored to measure cholinergic sensitivity. Females of each strain exhibited approximately 0.6 °C greater decreases in temperature than their male counterparts. The FSL rats were the most sensitive to OX, as expected, and the SD rats were intermediate between the FSL and FRL rats, confirming that both the FSL and FRL rats have diverged from normal SD rats. PYR, up to a dose of 12 mg/kg orally, did not produce a decrease in temperature in any of the 6 groups, confirming the lack of central effects. However, the FSL male rats appeared to be more sensitive to the heart rate lowering effects of PYR, a finding consistent with increased peripheral cholinergic sensitivity. Data on blood growth hormone levels, known to be stimulated by PYR, are being analyzed and will be reported at the meeting. These findings are consistent with the view that some humans may be genetically predisposed to exhibit greater sensitivity to PYR. (Supported by U.S. Army).

68

**MORE RAPID DECREASES IN TEMPERATURE FOLLOWING ANTICHOLINESTERASE CHALLENGES IN RATS PRETREATED WITH PYRIDOSTIGMINE.** DH Overstreet, Y Yang, E Clark, Jr., and AH Rezvani. *University of North Carolina School of Medicine, Chapel Hill, North Carolina*

Pyridostigmine bromide was given to Gulf War participants to protect them against nerve gas exposure. The present study sought to determine whether pyridostigmine will give equal protection to both genders of rat strains known to have innately different sensitivities to cholinergic agents and anticholinesterases. The male and female FSL (very sensitive), SD (outbred Sprague-Dawley obtained from Harlan) and FRL (resistant) rats were chronically treated with saline or pyridostigmine (12 mg/kg) by gavage for 14 days. Challenges with saline, chlorpyrifos (CPF, 60 mg/kg, by gavage), or diisopropylfluorophosphate (DFP, 1 mg/kg, s.c.) were given 30 min after the last treatment. Temperatures were monitored telemetrically during the last three days of chronic treatment and for at least 2 hr after the challenges. As expected, the FSL rats exhibited greater decreases in temperature than the FRL rats after both challenge agents. The SD rats tended to exhibit intermediate responses. For most of the groups the decrease in body temperature occurred at a more rapid rate in the rats pretreated with pyridostigmine. When the changes in temperature one hr after the challenges were compared with those immediately before the challenges, all pyridostigmine-treated groups except for the FSL males challenged with CPF exhibited significantly greater changes than their saline-treated counterparts. By the end of 2 hr, these differences had largely disappeared. These findings indicate that pyridostigmine certainly does not protect rats from the hypothermia induced by centrally acting anticholinesterase agents. The more rapid decrease in temperature in the pyridostigmine-treated groups may indicate that it is indeed protecting peripheral enzymes; however, a consequence of this "protection' seems to be a more rapid entry of CPF and DFP into the brain.

**189.9**

**WITHDRAWAL FROM A BINGE-LIKE DOSE OF *d*-AMPHETAMINE REDUCES MOTIVATION TO OBTAIN NATURAL REWARDS IN MALE RATS** A.M.Barr*, D.F.Fiorino and A.G.Phillips. Department of Psychology, University of British Columbia, BC, CANADA, V6T 1Z4.

.Psychostimulant withdrawal in humans is associated with numerous depressive-like symptoms, including anhedonia and anergia. Similar deficits have been found in rats subjected to psychostimulant withdrawal, assessed mostly by alterations in their responding for reinforcing intracranial self-stimulation of different brain sites. The current experiments used a binge-like dose of *d*-amphetamine to determine if withdrawal from this drug could be shown to decrease subjects' motivation to obtain natural rewards. In the first experiment, rats were trained to lever press under a progressive ratio schedule of reinforcement for a weak sucrose solution. After training, half of the rats were subjected to the *d*-amphetamine schedule, while the other half received vehicle. Withdrawal from the drug was associated with significantly reduced breakpoints, but free consumption of the solution remained unaffected. In the second experiment, male rats were trained to copulate with estrous female rats in modified bi-livel chambers, with a 5 minute precopulatory phase followed by unlimited access to the female for a subsequent 25 minutes. Following seven training sessions, half of the rats received the *d*-amphetamine schedule, and the other half received vehicle. Rats experiencing drug withdrawal showed reductions in several motivational components of sexual behaviour, including anticipatory locomotor activity and post-ejaculatory intervals, while most consummatory components remained unaffected. The results from these two experiments suggest that withdrawal from *d*-amphetamine in rats may be more closely associated with a reduction in their motivation to obtain natural rewards, rather than a decrease in their capacity to consume such rewards. *Funded by MRC Canada.*

**189.10**

SUPERSENSITIVE GROWTH HORMONE RESPONSES FOLLOWING PYRIDOSTIGMINE CHALLENGE IN A GENETIC ANIMAL MODEL OF DEPRESSION. D.H. Overstreet*, G.A. Mason, Y. Yang and A.H. Rezvani. Dept. Psychiatry, Univ. No. Carolina, Chapel Hill, NC 27599-7178.

Pyridostigmine is an anticholinesterase agent that has been widely used to study cholinergically induced growth hormone responses in humans and has also been widely prescribed as a prophylactic in soldiers deployed in the Persian Gulf War. One of the subgroups of humans who exhibit exaggerated growth hormone responses to pyridostigmine are individuals with major depression. It was predicted, therefore, that the Flinders Sensitive Line (FSL) rat, a genetic animal model of depression achieved by selective breeding for increased cholinergic responses, would exhibit greater growth hormone responses following a challenge with pyridostimine than the control Flinders Resistant Line (FRL) rat. Both sexes of the FSL, FRL and randomly bred Sprague-Dawley (SD, Harlan) rats were given either saline vehicle or 4, 12, or 36 mg/kg pyridostigmine by gavage and sacrificed by decapitation 30 min later. Telemetrically monitored temperature and activity measures, which did not differ, were reported at a previous meeting. The serum was analyzed for growth hormone using a kit from the NIDDK. Pyridostigmine produced in all groups an elevation of growth hormone that followed an inverted U-shape function, with highest levels being produced by the 12 mg/kg dose. The FSL and SD rats exhibited higher levels of growth hormone than the FRL rats at the doses of 4 and 12 mg/kg. Thus, these data confirm the cholinergic supersensitivity of the FSL rats using an endocrine challenge procedure which has reported a similar exaggerated response in depressed individuals. Supported by a contract from the U.S. Army.

**189.11**

ANTIPSYCHOTIC DRUGS INCREASE TYROSINE HYDROXYLASE IMMUNOREACTIVITY AND INDUCE CYTOPLASMIC GRANULES IN PC12 CELLS. O. Kontkanen*, G. Wong and E. Castrén. A.I.Virtanen Institute, University of Kuopio, P.O.Box 1627, FIN-70211 Kuopio, Finland.

Antipsychotic drugs are dopamine D2 receptor antagonists. The effect on D2 receptors is immediate, however, the clinical response takes several days or weeks to fully develope. We are interested in the effects of chronic antipsychotic treatment and use exposure to neuronal cell lines as a model. This study characterizes some effects of acute and chronic antipsychotic treatment on PC12 cells.

PC12 cells were plated on collagen coated plastic chamber slides and cultured in 15% serum in the presence of 0,1-10 µM chlorpromazine (CPZ) or differentiated with nerve growth factor (NGF, 50 ng/ml) in 1% serum. After various time periods of CPZ exposure (1-7d) the cells were fixed and stained with anti-tyrosine hydroxylase (TH) antibody. CPZ (1-10 µM) increased TH-immunoreactivity in both undifferentiated and differentiated cells after 3d. Differentiation of the cells alone did not affect the intensity of immunoreactivity. CPZ also induced the appearance of cytoplasmic granules within 36 hours of exposure to the drug. Formation of granules was not observed with 1 µM treatment, but showed dose dependence in their density with 3 µM and 10 µM application of CPZ. CPZ appeared to be toxic to PC12 cells after 7 days of exposure at the concentration of 10 µM. Granule formation was also observed with the exposure to 10 µM haloperidol for 5 days in culture.

Future experiments using primary neuronal cultures may help to understand the significance of cytoplasmic granules in antipsychotic drug action. This study is supported by the Academy of Finland and Sigrid Juselius foundation.

**189.12**

EFFECT OF PROPOFOL INFUSION ON TYROSINE HYDROXYLASE IN THE NUCLEUS ACCUMBENS. H.C. Gray[1], P.A. Merrifield[1], A. Gelb[2], D.F. Cechetto[1]*, Depts. of Anatomy and Cell Biology[1] and Anaesthesiology[2]; Univ. Western Ontario; London Health Sciences Center; London, Ontario, Canada, N6A 5C1.

Propofol (2,6 diisopropylphenol) is an intravenous anaesthetic used for both the induction and maintenance of general anesthesia. It has many non-anesthetic actions, including cardiovascular depression, antiemetic and neuroexcitatory effects as well as behavioral changes, which may be due to effects on specific neurotransmitter systems. Previous studies using immunohistochemistry indicate a significant decrease in tyrosine hydroxylase (TH) in the nucleus accumbens (Acb) three days following a six hour, sub-anaesthetic, propofol infusion. In addition, rats receiving propofol exhibit a decrease in amphetamine stimulated rearing, a behavior shown to be mediated by dopamine release in the Acb. We used Western Blot to quantify the difference in TH levels in male Wistar rats infused with propofol or with Intralipid® (control). For comparison, experiments using isoflurane and the appropriate control group were also carried out. Three days post-infusion the animals were decapitated, the brains removed and frozen, and micropunches of neural tissue were obtained from the Acb, the caudate putamen, the lateral frontal cortex, and the insular cortex. Proteins were separated using SDS-PAGE, electrophoretically transfered onto nitrocellulose paper and TH localized using immunochemistry with a chemiluminescent substrate. Densitometry was used to quantify TH. The results indicate a 13-85% reduction in TH levels in the Acb of animals receiving propofol anesthesia compared to the Intralipid® control group. No change was observed in the other brain regions. A reduction in TH in the Acb suggests a decrease in the activation of the dopaminergic system consistant with the behavioral results. Overactivity of the dopaminergic system in the mesolimbic system is believed to be involved in the manifestation of schizophrenic symptoms. A reduction in TH provides evidence that sub-anaesthetic doses of propofol may be an effective anti-psychotic agent.

**189.13**

ADENOVIRAL GENE TRANSFER OF CNTF AND NAIP CAN RESCUE MOTONEURONS AFTER NEONATAL SCIATIC NERVE AXOTOMY. D. Perrelet[1], Y. Sagot[1], A. MacKenzie[2], G.M. Smith[3] and A.C. Kato[1]*.
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Neurotrophic factors and anti-apoptosis molecules have been shown to afford neuroprotection to injured rodent motoneurons following neonatal axotomy. In most of the studies to date using this experimental paradigm, the factors were applied as a single bolus directly onto the lesioned nerves and their rescue effects were found to decline at time-points beyond one week. The goal of our experiments was to determine whether administration of neurotrophic factors (CNTF and BDNF) and NAIP (neuronal apoptosis inhibitory protein) via adenoviral gene transfer could rescue motoneurons on a long-term basis. Adenoviral vectors were applied onto the proximal sciatic nerve stump in 4 day old rats by a capping technique ($10^6$ to $2.25x10^7$ pfu in a volume of 3 µl). The fluorescent tracer, fluorogold was simultaneously administered as a means for quantitatively assessing the rescue of fluorescently-labelled motoneurons in serial sections of the lumbar spinal cord. Control experiments using an adenovirus expressing the lacZ gene showed that 13% of the sciatic motoneuron pool was transfected. After 1 week survival, Adv-CNTF, Adv-BDNF and Adv-NAIP were all capable of rescuing 26 % of the motoneurons, whereas at 2 weeks, only vectors with CNTF and NAIP were effective (41 % as compared to control). At 3 weeks, none of these agents were active probably due to a decrease in transgene expression inside the target neurons. In addition, adenoviral vectors were administered into the gastrocnemius muscle 3 days prior to axotomy; this "pre-treatment" paradigm did not increase motoneuron rescue. (supported by the Swiss National Science Foundation and the French Muscular Dystrophy Association)

**189.14**

ALTERATIONS IN NEUROPEPTIDE Y AND Y1 RECEPTOR mRNA EXPRESSION IN BRAINS FROM AN ANIMAL MODEL OF DEPRESSION: REGION SPECIFIC ADAPTATION AFTER FLUOXETINE TREATMENT. L. Caberlotto*, K. Fuxe, D. H. Overstreet, P. Gerrard, and Y.L. Hurd. Karolinska Institute, Depts. of Neuroscience and Clinical Neuroscience, Stockholm, Sweden, Dept. of Psychiatry, University of North Carolina, Chapel Hill, NC,USA, Dept. Pharmacology, Glaxo-Wellcome, Verona, Italy.

To investigate the possible link between NPY and depression, we analyzed NPY and its receptors in different limbic-related regions in the Flinder sensitive Line (FSL), a genetic animal model of depression. NPY and NPY receptors, Y1 and Y2, mRNA expression levels were measured in the FSL as compared to the control Flinder resistant Line rats (FRL). In the FSL rats, NPY mRNA expression levels were significantly decreased in the nucleus accumbens and CA regions, but increased in the arcuate nucleus and anterior cingulate cortex. Y1 receptor mRNA expression was decreased in different cortical areas and in the hippocampal dentate gyrus. Y2 mRNA expression levels did not differ between FSL and FRL animals. The antidepressant drug fluoxetine (a serotonin reuptake inhibitor; 10 µmoles/kg; daily for 14 days) caused an increase of the NPY mRNA hybridization signal in the arcuate nucleus of both strains. In other brain regions, fluoxetine administration caused a differential effect on the induction of NPY-related genes in the two rat strains: in the CA region and dentate gyrus NPY mRNA expression was increased in the FSL, but decreased in the FRL. In contrast, Y1 mRNA levels tended to be decreased by fluoxetine in the nucleus accumbens of the FSL rats, but increased in the FRL. These findings give support to the hypothesis of an impairment of NPY in depression and also suggest an involvement of the Y1 receptor gene. Investigations are being carried out to assess the correlation between mRNA levels, protein content, and binding sites at the pre- and post-junctional levels of NPY neurons in the Flinders animal model.

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# FAILURE OF PYRIDOSTIGMINE PRETREATMENT TO PROTECT AGAINST THE EFFECTS OF CHLORPYRIFOS

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Soldiers who were deployed to the Persian Gulf War were commonly given pyridostigmine, a peripherally acting anticholinesterase (anti-ChE) agent, to protect against exposure to nerve agents, which are centrally acting anti-ChEs. The present project was designed to test this strategy in rat strains which are known to be differentially sensitive to anti-ChEs and other cholinergic agents. Both sexes of the Flinders Sensitive Line (FSL) rats, which are more sensitive to cholinergic agents, their selectively bred counterparts, the Flinders Resistant Line (FRL) rats, and randomly bred Sprague-Dawley (SD) rats were first challenged with oxotremorine (0.2. mg/kg), a centrally acting cholinergic agonist, to confirm gender and strain differences. Oxotremorine-induced hypothermia was influenced by both gender and strain: the female rats exhibited a greater degree of hypothermia than their male counterparts, and the FSL rats exhibited a greater degree of hypothermia than their FRL counterparts, with the SD rats having intermediate scores.

Within 24 hours of the oxotremorine challenge, the rats began being treated chronically with pyridostigmine (12 mg/kg by gavage) or saline vehicle for 14 days. On the day of the last treatment the rats received an acute treatment of chlorpyrifos (CPF, 60 mg/kg by gavage 30 min after last chronic treatment). Body temperature and activity were recorded telemetrically via previously implanted transmitters. CPF had both strain- and gender-dependent effects and these effects interacted with pyridostigmine pretreatment. Female rats were more sensitive to the hypothermic effects of CPF than their male counterparts, as were the FSL rats compared to the FRL rats. Pyridostigmine did not alter the effects of CPF in the more resistant male rats, but potentiated its effects in the female rats. Group differences for the activity measures were not as pronounced. Thus, the more sensitive female rats, instead of being protected by the pyridostigmine pretreatment, were more sensitive to CPF.

These findings provide no support for the strategy of pretreating individuals with pyridostigmine as a protection against nerve gas exposure and suggest that the strategy may make certain individuals more sensitive to these agents.

**KEY WORDS: Pyridostigmine, Flinders Rats, Chlorpyrifos**